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Studies on the Roles of ATP in Nitrogenase Catalysis

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STUDIES ON THE ROLES OF ATP IN NITROGENASE CATALYSIS

by

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ABSTRACT

Studies on the Roles of ATP in Nitrogenase Catalysis

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Nitrogenase is the enzyme that catalyzes the reduction of nitrogen to ammonia in a reaction requiring MgATP hydrolysis. Two component proteins of nitrogenase are the iron protein (Fe protein) and the molybdenum-iron protein (MoFe protein).

Nitrogenase contains two nucleotide binding sites. During catalysis, the Fe protein binds two MgATP first. The conformational changes induced upon MgATP binding allow the Fe protein to associate with the MoFe protein. After the formation of the Fe protein-MoFe protein complex, a single electron is transferred from the Fe protein to the MoFe protein, an event that is coupled to MgATP hydrolysis in the Fe protein. The wild-type Fe protein and all the altered Fe proteins studied so far are homodimeric. In order to assess the contribution of each nucleotide binding site in the

Fe protein to the events occurring during nitrogenase catalysis, a heterodimeric Fe protein was constructed that has Asp 39 substituted by Asn in one subunit and the other subunit the same as the wild-type Fe protein. Characterization of this heterodimeric Fe protein showed that alterations in the properties of the [4Fe-4S] cluster that occur upon nucleotide binding to the Fe protein are due to the additive effect of each nucleotide binding to the Fe protein. The rates of MgATP hydrolysis and MgATP-dependent primary electron transfer of this heterodimeric Fe protein are intermediate between those of the homodimeric wild-type Fe protein and D39N Fe protein. These observations suggested that each ATP binding site contributes to the rate acceleration of primary electron transfer. After electron transfer, this heterodimeric Fe protein forms a tight complex with the MoFe protein, demonstrating that alteration in one subunit is enough for the formation of a tight nitrogenase complex. When this heterodimeric Fe protein was combined with the MoFe protein, no substrate reduction was detected. Therefore, two functional subunits of the Fe protein are necessary for reduction of substrates.

The mechanism of ATP hydrolysis in the Fe protein was also investigated. Using site-directed mutagenesis, the role of lysine 10 of the *Azotobacter vinelandii* nitrogenase Fe protein in MgATP hydrolysis was examined. Changing Lys 10 of the protein to Arg resulted in an Fe protein that hydrolyzed MgATP at a rate 3% that of the

wild-type Fe protein. The affinities of the K10R Fe protein for nucleotides and the changes in the properties of the [4Fe-4S] cluster of the K10R Fe protein upon nucleotide binding were compared with those of the wild-type Fe protein. These results indicated that in the absence of the MoFe protein, the interactions of the K10R Fe protein with nucleotides are similar to the wild-type Fe protein. After the Fe protein-MoFe protein complex formation, the dramatic decrease in the rate of MgATP hydrolysis of the K10R Fe protein indicated a role of Lys 10 in ATP hydrolysis. This conclusion is consistent with the X-ray crystal structure of the nitrogenase complex stabilized by the AlF_4^-•ADP, where Lys 10 is proposed to facilitate product formation in ATP hydrolysis.

(114 pages)

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Wei Wu

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[Asn^{39}/Asn^{39}] Fe protein	Fe protein with Asn at position 39 on both subunits
[Asp^{39}/Asn^{39}] Fe protein	Fe protein with one subunit containing Asp at position 39 and the other subunit containing Asn at position 39
[Asp^{39}/Asp^{39}] Fe protein	wild-type Fe protein with Asp at position 39 on both subunits
BPS	bathophenanthrolinedisulfonic acid
CD	circular dichroism
D39N Fe protein	Fe protein with Asp 39 substituted by Asn on both subunits, the same expression as [Asn^{39}/Asn^{39}] Fe protein
EPR	electron paramagnetic resonance
Fe Protein	iron protein of nitrogenase
GppNHp	GTP with the bridging oxygen between the β and γ Phosphates substituted by NH
GTPγS or ATPγS	GTP or ATP with one oxygen atom of the γ-phosphate substituted with sulfur atom
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

^{1}H NMR	proton nuclear magnetic resonance
IDS	indigo disulfonate
L127Δ Fe protein	Fe protein with Leu 127 deleted on both subunits
MoFe protein	molybdenum-iron protein of nitrogenase
MOPS	3-(N-morpholino)propanesulfonic acid
Tris	tris-(hydroxymethyl)aminomethane
XppY Fe protein	general expression for an altered Fe protein, representing an Fe protein with X (single letter abbreviation for amino acid) substituted by Y (single letter abbreviation for amino acid) on both subunits at the position of pp (amino acid number)

CHAPTER 1

LITERATURE REVIEW

All organisms need nitrogen as a constituent of proteins and nucleic acids. The N_2 in the atmosphere cannot be directly utilized by most organisms for their metabolism or biosynthesis. In chemical and biological nitrogen fixation, atmospheric N_2 is converted to ammonia, which then can be used by many organisms.

The most common method of industrial ammonia synthesis is called the Haber-Bosch process. High temperature (600-800K) and high pressure (500 atm) are applied in order to get the suitable yield of ammonia from N_2 and H_2. In contrast to the chemical ammonia synthesis, biological nitrogen fixation occurs at very mild temperature and under the pressure of about 1 atm. Biological nitrogen fixation comprises more than 50% of the nitrogen fixation in the entire world. Due to the importance and efficiency of biological nitrogen fixation, the mechanism of this process has been studied extensively.

The ability of some prokaryotic organisms to fix N_2 is provided by nitrogenase systems existing in them. Nitrogenase in the nitrogenase system is the enzyme that catalyzes the reduction of N_2 to ammonia. Several kinds of nitrogenases exist in nature (Eady, 1996). These include the iron-only nitrogenase, the vanadium-containing

nitrogenase, and the molybdenum-containing nitrogenase, which will be the focus of this literature review.

The molybdenum nitrogenase in *Azotobacter vinelandii* has two component proteins, the iron protein (Fe protein) and the molybdenum iron protein (MoFe protein). Figure 1-1 shows the molecular model of the MgADP•AlF_4^- stabilized nitrogenase complex. The Fe protein is a 64 kDa homodimer with two nucleotide binding sites, one in each subunit, and a [4Fe-4S] cluster located at the opposite end of the Fe protein relative to the nucleotide binding sites (Georgiadis et al., 1992). The MoFe protein is a 250 kDa $\alpha_2\beta_2$ tetramer with two pairs of unique metal clusters called the P-cluster and the FeMo cofactor. The P-cluster is an [8Fe-7S] cluster bridging the α and β subunits of the MoFe protein (Kim & Rees, 1992). The FeMo cofactor is a [1Mo-7Fe-9S-homocitrate] cluster within the α subunit of the MoFe protein. The P-cluster is believed to mediate electron transfer from the [4Fe-4S] cluster of the Fe protein to the FeMo cofactor where substrate reduction occurs (Chan et al., 1999a; Lowe et al., 1993; Ma et al., 1996; Peters et al., 1995; Shah & Brill, 1977).

REDOX STATES OF THE METAL CLUSTERS OF THE NITROGENASE

The metal clusters of the nitrogenase undergo redox changes during nitrogenase turnover. In the presence of dithionite, the [4Fe-4S] cluster of the Fe protein is in the

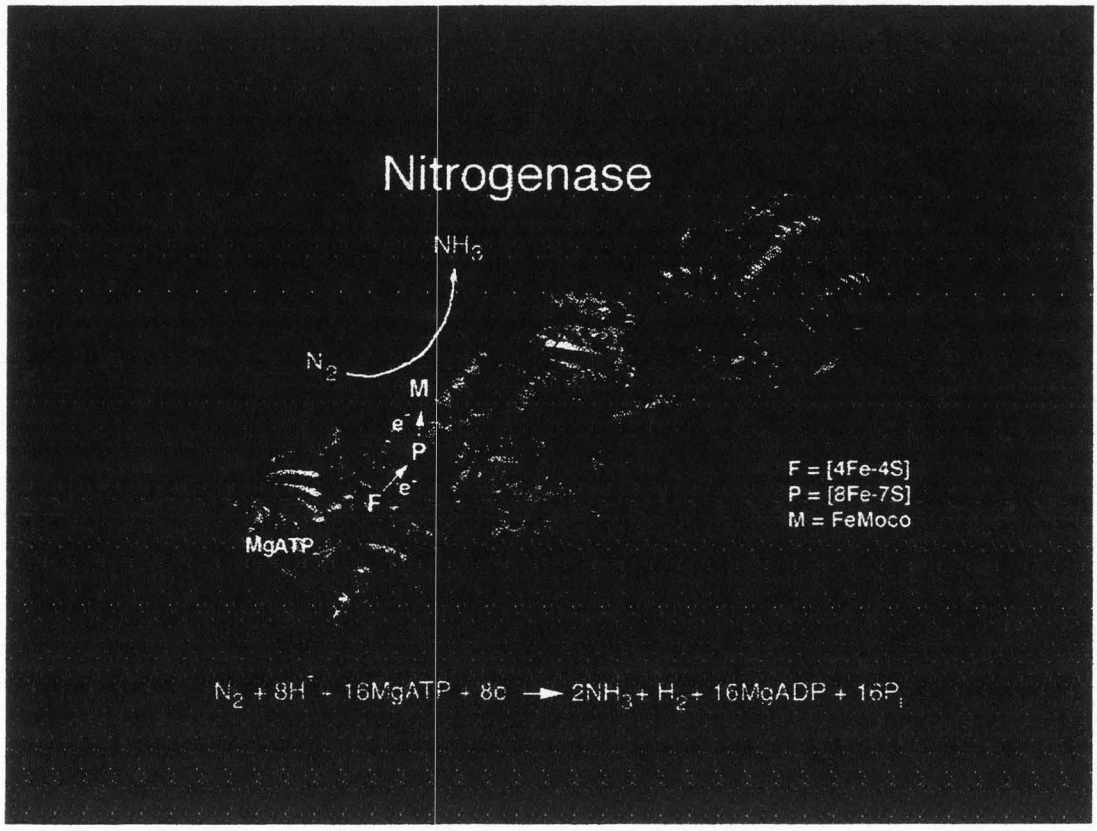

FIGURE 1-1: A molecular model of the nitrogenase complex stabilized by MgADP•AlF_4^-. The Fe proteins, and α and β subunits of the MoFe protein are shown in yellow, dark blue, and cyan, respectively. The four bound MgADP•AlF_4^- molecules in the Fe protein are shown in magenta. The [4Fe-4S] clusters, P-clusters, and FeMo cofactors are shown in red. The pathway for electron flow within the nitrogenase is denoted by white arrows. The overall reaction for the reduction of nitrogen by nitrogenase is shown at the bottom. This model was generated based on the crystal structure of the MgADP•AlF_4^- stabilized nitrogenase complex (Schindelin et al., 1997), using the programs MOLSCRIPT (Kraulis, 1991) and Ratser3D (Merrit & Murphy, 1994).

1+ oxidation state. Upon electron transfer from the Fe protein to the MoFe protein, the oxidation state of the [4Fe-4S] cluster changes from the 1+ to the 2+ state. Recently, a $[4Fe\text{-}4S]^0$ state, or all-ferrous state, of the [4Fe-4S] cluster was reported when methyl viologen or flavodoxin was used as the reductant (Watt & Reddy, 1994). It is possible that the Fe protein in the $[4Fe\text{-}4S]^0$ state could transfer two electrons to the MoFe protein during one catalytic cycle. The oxidation states of the [4Fe-4S] cluster under physiological conditions are still not clear.

Several oxidation states of the P-cluster have been shown (Burgess & Lowe, 1996). In the presence of dithionite, the P-cluster of the MoFe protein is in the P^N state. One electron oxidation of the P-cluster in P^N state leads to the P^{1+} state. Further oxidation leads to the P^{2+} state. It is also known the FeMo cofactor can achieve several oxidation states and it becomes more reduced during nitrogenase turnover than the FeMo cofactor in the as-isolated MoFe protein with dithionite present.

NITROGENASE CYCLE

Nitrogenase catalyzes reduction of substrates in a reaction that needs the energy of MgATP hydrolysis (Burris, 1991). The generally accepted nitrogenase catalytic cycle is as follows. During catalysis, the Fe protein binds two MgATP molecules, which induce conformational changes in the Fe protein and also allow the Fe protein to

bind to the MoFe protein (Seefeldt, 1997). After Fe protein-MoFe protein complex formation, a single electron is transferred from the Fe protein to the MoFe protein in a process that is coupled to MgATP hydrolysis in the Fe protein (Burgess & Lowe, 1996; Seefeldt, 1997). The MgADP-bound Fe protein then dissociates from the MoFe protein. The oxidized Fe protein is reduced by flavodoxin or ferrodoxin, and two MgADP are replaced by two MgATP in the Fe protein (Thorneley & Lowe, 1985). The Fe protein is now ready to associate with the MoFe protein again. This cycle needs to be repeated until enough electrons accumulate within the MoFe protein to reduce the substrate (Thorneley & Lowe, 1984).

BINDING OF MGATP OR MGADP TO THE FE PROTEIN

Nucleotides play an important role in nitrogenase catalysis. Fe protein binds nucleotides with strong positive cooperativity (Cordewener et al., 1985). The affinities of the Fe protein for nucleotides have been determined by different techniques. The dissociation constants determined for MgATP and MgADP binding to the Fe protein vary dramatically depending on whether or not the technique directly measures the binding or measures the changes to the Fe protein after the nucleotide binding (Yates, 1992). An equilibrium column binding technique has been used to determine the affinity of the Fe protein for nucleotides. The dissociation constant for MgATP binding

to the Fe protein is determined to be around 560 μM and the value determined for MgADP is approximately 120 μM (Ryle et al., 1995). Regardless of the different constants determined for nucleotide binding to the Fe protein, two conclusions can be drawn. First, the Fe protein binds MgADP more tightly than MgATP in both the oxidized and the reduced states. Second, the oxidized Fe protein has greater affinities for MgATP and MgADP than the reduced Fe protein (Cordewener et al., 1985). The change in the oxidation state of the [4Fe-4S] cluster of the Fe protein results in a change in the affinity of the Fe protein for nucleotides. Given that the distance of the nucleotide binding site and the [4Fe-4S] cluster is 15 Å, the oxidation state of the [4Fe-4S] cluster must somehow affect the protein conformation, which then influences the affinity of the Fe protein for nucleotides.

The X-ray crystal structures of the Fe protein alone and the Fe protein-MoFe protein complex stabilized by AlF_4^-•ADP provided information on the nucleotide binding modes in the Fe protein (Georgiadis et al., 1992; Schindelin et al., 1997). The crystal structures combined with characterization of altered Fe proteins allow us to elucidate the roles of specific residues in nucleotide binding to the Fe protein. Like other nucleotide binding proteins, the Fe protein of nitrogenase contains two nucleotide binding motifs, specified as Walker A and Walker B motifs (Walker et al., 1982). The Walker A sequence in the Fe protein includes residues Gly 9 to Ser 16. The Walker B

sequence includes residues Glu 125 to Gly 128 (Robson, 1984). Several residues in the Walker A and Walker B motifs are directly involved in nucleotide binding. Figure 1-2 shows the interactions of the Fe protein with the bound ADP · AlF_4^- in the crystal structure of the nitrogenase complex. In the crystal structure of the Fe protein alone, a salt bridge exists between Lys 15 in the Walker A motif and Glu 125 in the Walker B motif. In comparison, the crystal structure of the AlF_4^-•ADP stabilized Fe protein-MoFe protein complex shows that Lys 15 interacts with the phosphate moiety of the nucleotide and Asp 125 may coordinate with the Mg^{2+} through a bound water. This means that the salt bridge must be broken when nucleotides bind to the Fe protein. An analysis of an altered Fe protein with Lys 15 substituted with Arg (K15R Fe protein) showed that this protein is not able to bind nucleotides (Ryle et al., 1995). One explanation for this observation is that Arg 15 probably forms a stronger salt bridge with Glu 125 and cannot be broken, which prevents the insertion of the nucleotide between these two amino acid residues. Characterization of D125E-altered Fe protein also suggested a role for this residue in nucleotide binding (Wolle et al., 1992a). The X-ray crystal structure of the nitrogenase complex also showed that Ser 16 is coordinated with the Mg^{2+}, which interacts with the β-phosphate and two F ligands of AlF_4^-. Studies of the Fe proteins that have Ser 16 replaced by other amino acids

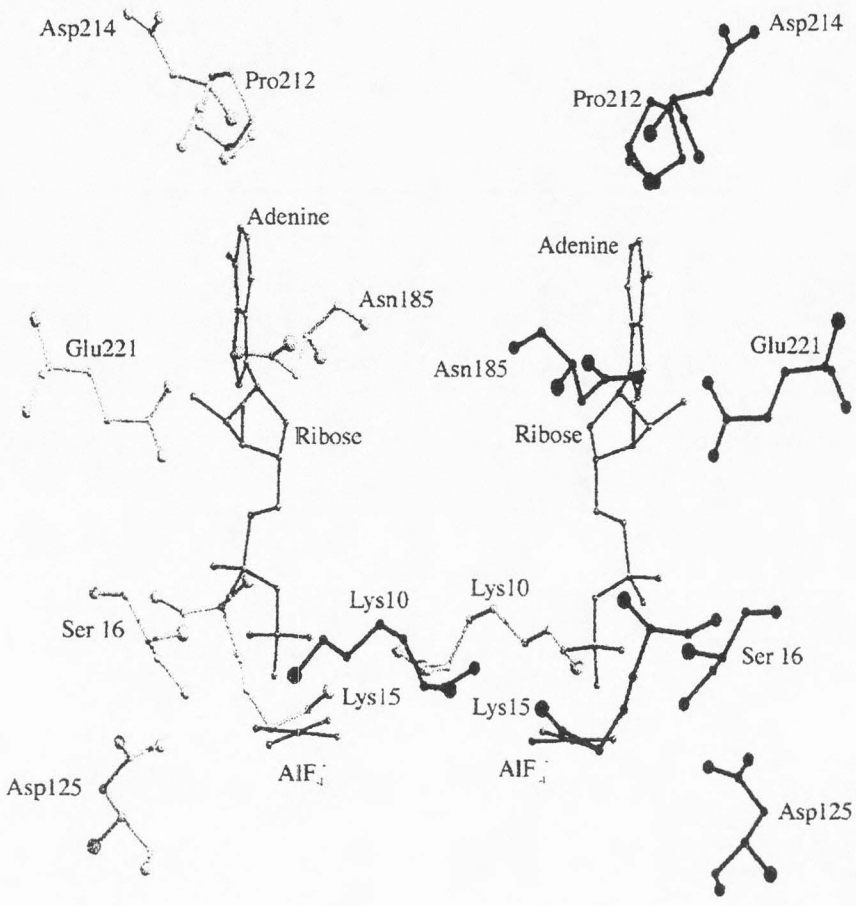

FIGURE 1-2: Interactions of the A. *vinelandii* Fe protein with the bound ADP•AlF_4^- in the crystal structure of the nitrogenase complex (Schindelin et al., 1997). The residues from one subunit of the Fe protein are shown in dark color while the corresponding residues from the other subunit are shown in light color. All the residues and the ADP · AlF_4^- are labelled and shown as ball-and-stick models. This graph was generated using the program MOLSCRIPT (Kraulis, 1991) and Ratser3D (Merrit & Murphy, 1994).

indicated that Ser 16 is important in the binding Mg^{2+} of MgATP or MgADP (Seefeldt & Mortenson, 1993). The side chain oxygen of Thr 17 and the peptide amide of Gly 128 also interact with the bound GDP• AlF_4^- in the crystal structure.

Two binding modes have been suggested for the binding of the ribose and adenine portion of the nucleotide (Wolle et al., 1992a). A cross-subunit binding mode has been proposed based on the crystal structure of the Fe protein alone, where the adenine portion of the bound ADP crosses the subunit interface and interacts with amino acid residues in the other subunit. The second binding mode is called the Ras-like binding mode and is based on the binding of GTP in p21ras. The crystal structure of the nitrogenase complex stabilized by AlF_4^-•ADP revealed ADP binding to the Fe protein in a Ras-like binding mode as shown in Figure 1-2, where each nucleotide is in contact primarily with one monomer of the Fe protein (Schindelin et al., 1997). The side chain of Glu 221 is hydrogen bonded to the O2' and O3' of the ribose. The N1 of the adenine portion of the nucleotide is hydrogen bonded with the peptide amide of Asp 214, and the exocyclic amino group of the adenine interacts with the side chain of Asn 185 and the amide oxygen of Pro 212. These interactions probably determine the specificity of the nitrogenase for ATP in substrate reduction.

NUCLEOTIDE-INDUCED CONFORMATIONAL CHANGES IN THE FE PROTEIN

Upon nucleotide binding to the Fe protein, conformational changes induced in the Fe protein can be reflected as changes in the spectroscopic properties of the [4Fe-4S] cluster. The [4Fe-4S] cluster of the Fe protein is covalently bound by Cys 97 and Cys 132 from two subunits. In addition, the cluster is also in van der Waals contact with Ala 98, Val 130, and Phe 135 (Georgiadis et al., 1992). It is expected that nucleotide-induced conformational changes in the Fe protein affect the protein environment around the [4Fe-4S] cluster, thus changing the properties of the [4Fe-4S] cluster.

Electron paramagnetic resonance (EPR) is used as a tool to detect the state of unpaired electrons in the [4Fe-4S] cluster of the Fe protein. The $[4Fe\text{-}4S]^{+}$ cluster of the reduced Fe protein demonstrates a rhombic EPR signal with g values around 2. The addition of MgATP to the reduced Fe protein results in a change in the EPR signal from rhombic to axial (Orme-Johnson et al., 1972; Smith et al., 1973; Zumft et al., 1973). In addition, the EPR spectrum of the Fe protein with MgADP bound is slightly different from that of the Fe protein without nucleotides bound. Besides differences in EPR signals, it has also been shown that the circular dichroism (CD) spectrum of the Fe protein in the absence of nucleotides is distinct from that of the Fe protein in the

presence of nucleotides. CD spectroscopy monitors changes in the protein environment around the [4Fe-4S] cluster of the protein (McKenna et al., 1984; Stephens et al., 1978; Stephens et al., 1979; Stephens et al., 1981). Early CD studies compared nitrogenase from different diazotrophic organisms, and all showed similar spectra, independent of the nitrogenase source. These early studies also showed that there was no difference between the CD spectrum of the Fe protein with MgATP bound and that of the Fe protein with MgADP bound. Recently, however, it was found that the CD spectrum of the Fe protein with MgATP is distinct from the spectrum of the Fe protein with MgADP, providing further evidence that the conformations of the Fe protein with MgATP and MgADP bound are different (Ryle et al., 1996). ^{1}H NMR spectroscopy also reflects changes in the properties of the [4Fe-4S] cluster (Lanzilotta et al., 1995a; Meyer et al., 1988). The paramagnetically shifted resonances arise from the α-CH and β-CH_2 protons of cysteinyl ligands of the [4Fe-4S] cluster of the Fe protein (Lanzilotta et al., 1995a). Upon addition of the nucleotides, the ^{1}H NMR signals are shifted. Comparing the CD, EPR, and ^{1}H NMR spectra of the wild-type Fe protein and the altered Fe protein can provide some information on whether or not the residue substituted in the altered Fe protein is involved in the nucleotide-induced conformational change.

Another event that occurs upon MgATP binding to the Fe protein is the

accessibility of the iron of the [4Fe-4S] cluster to chelators. In the absence of nucleotides, the iron of the [4Fe-4S] cluster is not easily accessible. In the presence of MgATP, but not MgADP, the iron of the [4Fe-4S] cluster is readily chelated by compounds such as bathophenanthrolinedisulfonate (BPS) and α,α'-dipyridyl (Deits & Howard, 1989; Ljones & Burris, 1978; Walker & Mortenson, 1974). It is suggested that conformational changes induced in the Fe protein upon MgATP binding cause the [4Fe-4S] cluster of the Fe protein to be more solvent accessible, which allows chelators to access the iron. The addition of the MoFe protein to chelation assays slows the rate of chelation (Walker & Mortenson, 1974). This observation could be due to protection by the MoFe protein by blocking access to the [4Fe-4S] cluster or to an additional conformational change induced in the Fe protein by the MoFe protein. Although MgADP is also known to cause a conformational change in the Fe protein, no iron from the [4Fe-4S] cluster of the wild-type Fe protein is chelated by BPS or α,α'-dipyridyl in the presence of the MgADP. This also suggests that the conformational changes induced in the Fe protein by MgATP or MgADP are unique.

Further evidence that nucleotides induce Fe protein conformational changes is provided by the change in the redox potential of the [4Fe-4S] cluster upon nucleotides binding to the Fe protein (Watt et al., 1986; Zumft et al., 1974). In the absence of nucleotides, the midpoint potential of the $[4Fe\text{-}4S]^{2+/+}$ cluster of *A. vinelandii* Fe protein

is around -300 mV relative to the normal hydrogen electrode (NHE) as determined by redox titration. Binding of MgADP or MgATP to the Fe protein results in a decrease in the midpoint potential of the [4Fe-4S] cluster by over 100 mV. The midpoint potential of the $[4Fe\text{-}4S]^{2+/+}$ couple of the Fe protein with MgATP bound or with MgADP bound is about -420 mV or -460 mV, respectively (Lanzilotta et al., 1995b).

It seems reasonable to believe that the decrease in the midpoint potential of the [4Fe-4S] cluster will provide a stronger driving force for electron transfer from the [4Fe-4S] cluster of the Fe protein to the P-cluster of the MoFe protein. The MgADP bound Fe protein cannot transfer electron to the MoFe protein although it has lower midpoint potential than the MgATP bound Fe protein, so the decrease in the midpoint potential is not the only prerequisite for electron transfer to occur.

COMMUNICATION BETWEEN THE NUCLEOTIDE BINDING SITE AND THE [4FE-4S] CLUSTER OF THE FE PROTEIN

Nucleotide binding to the Fe protein transduces a distance of 15 Å to result in the changes of the properties of the [4Fe-4S] cluster. One shortest pathway for communication between the nucleotide binding region and the [4Fe-4S] cluster of the Fe protein is provided by switch II, which includes residues 125 to 132 (Howard & Rees, 1994; Ryle & Seefeldt, 1996). Figure 1-3 shows the position of switch II in the

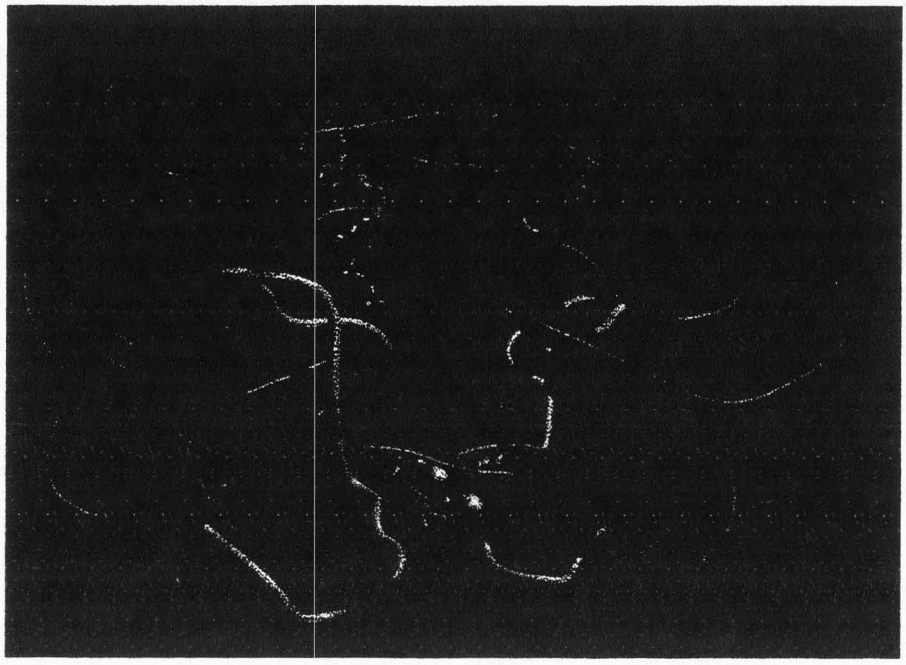

FIGURE 1-3: A molecular model of the nitrogenase Fe protein from *A. vinelandii*. The [4Fe-4S] cluster, which is in green and yellow spheres, is bridging the two subunits of the Fe protein. MgATP (ball-and-stick) has been modeled into the structure to illustrate the nucleotide binding site of the Fe protein. Switch I and switch II are shown in cyan and magenta, respectively. This model was generated based on the crystal structure of the Fe protein (Georgiadis et al., 1992), using the programs MOLSCRIPT (Kraulis, 1991) and Ratser3D (Merrit & Murphy, 1994).

Fe protein. Asp 125 of switch II is located in the nucleotide binding site of the Fe protein. As nucleotide binding to the Fe protein breaks the salt bridge between D125 and K15, it seems reasonable that a change in the position of D125 could result in a movement of switch II, subsequently changing the protein environment around the [4Fe-4S] cluster. In support of this, deletion of Leu 127 in switch II resulted in an Fe protein (L127Δ) that adopted a MgATP bound conformation in the absence of nucleotides, and formed a non-dissociating complex with the MoFe protein (Ryle & Seefeldt, 1996b).

COMPLEX FORMATION

Chemical linking reagent, 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide, has been used to study the complex formation. Using this reagent, the Fe protein and the MoFe protein are cross linked, even in the absence of nucleotides (Willing et al., 1989). The amino acids involved in the cross linking reaction include D112 of the Fe protein and K399 of the β-subunit of the MoFe protein (Willing & Howard, 1990). Although these two amino acids are specifically involved in the cross linking reaction, they are not conserved in either the Fe protein or the MoFe protein. It should be noted that addition of MgATP or MgADP does not affect the rate or the amount of the product of cross linking reaction. This result is surprising as it has been known that binding of

nucleotides to the Fe protein induces the protein conformational changes, which may affect complex formation. There is evidence to support that the affinity of the Fe protein for the MoFe protein indicated by the cross linking experiments does not reflect the possibility of the formation of the nitrogenase complex during biological conditions. An altered Fe protein, K15Q Fe protein, which is not able to associate efficiently with the MoFe protein based on a competitive binding assay with the wild-type Fe protein, can form the normal cross linking complex with the MoFe protein (Seefeldt et al., 1992). It was found that the K15Q Fe protein still binds MgATP with reduced affinity, but it could not undergo the proper MgATP-induced conformational changes. In fact, the MgATP-induced conformational changes in the Fe protein are thought to be necessary for complex formation between the Fe protein and the MoFe protein.

RESIDUES AND REGION THAT ARE INVOLVED IN COMPLEX FORMATION

The association of the Fe protein with the MoFe protein probably involves electrostatic interactions between the charged amino acids on the Fe protein and the charged amino acids on the MoFe protein. This conclusion is based on the fact that the interaction of the Fe protein with the MoFe protein is very sensitive to salt concentrations (Deits & Howard, 1990). Using site-directed mutagenesis, some specific

residues and regions that participate in the Fe protein-MoFe protein association have been identified. Arg 100, Arg 140, and Lys 143 are suggested to be involved in complex formation between the Fe protein and the MoFe protein (Lowery et al., 1989; Seefeldt, 1994; Wolle et al., 1992b). Characterization of the R100Y, R100H, R140Q, and K143Q Fe proteins showed that these altered Fe proteins have decreased affinities for the MoFe protein. In addition, one region in the Fe protein that is believed to be involved in complex formation is defined by residues 59 to 67 (Howard & Rees, 1994). Previously, it was shown that the *Clostridium pasteurianum* Fe protein could form a tight complex with the *Azotobacter vinelandii* MoFe protein (Emerich et al., 1978). The replacement of the residues 59 to 76 of the *A. vinelandii* Fe protein by the corresponding residues in the *C. pasteurianum* Fe protein resulted in an Fe protein that forms a tighter complex with the *A. vinelandii* MoFe protein than the wild-type *A. vinelandii* Fe protein (Peters et al., 1994).

COMMUNICATION BETWEEN THE NUCLEOTIDE BINDING SITE AND THE FE PROTEIN-MOFE PROTEIN DOCKING INTERFACE

The nucleotide binding site in the Fe protein is about 17 Å from the Fe protein-MoFe protein docking surface. How does the binding of MgATP to the Fe protein increase the affinity of the Fe protein for the MoFe protein? Switch I, which is shown

in the Figure 1-3, may be involved in this communication pathway. Switch I connects the nucleotide binding site and the Fe protein-MoFe protein interface (Howard & Rees, 1994; Lanzilotta et al., 1997a) and includes residues 39 to 67. Asp 39 is located in close proximity to the nucleotide binding site. Residues 59 to 67 form a loop near the Fe protein-MoFe protein docking surface and have been shown to be involved in the association of the Fe protein with the MoFe protein (Peters et al., 1994). Upon nucleotide binding to the Fe protein, partial protein conformational changes induced around the nucleotide binding site could be communicated to the Fe protein-MoFe protein docking surface through switch I. So far there are no experimental data to support that switch I is involved in the association of the Fe protein with the MoFe protein or MgATP induced conformational changes. Characterization of an Fe protein with Asp 39 replaced by Asn showed that switch I plays a role in the dissociation of the Fe protein from the MoFe protein and MgADP induced conformational changes (Lanzilotta et al., 1997a). It is possible that switch I mediates two-way communication between the nucleotide binding site and the Fe protein-MoFe protein docking surface.

STRUCTURAL CHANGES UPON COMPLEX FORMATION

Upon nitrogenase complex formation, no large structural changes have been detected for the wild-type MoFe protein by comparison of the crystal structures of the

MoFe protein in the uncomplexed and the complexed form. However, significant structural changes have been observed for the wild-type Fe protein (Schindelin et al., 1997). Upon complex formation, each Fe protein subunit rotates 13° towards the subunit interface, which results in a more compact conformation of the Fe protein. The new dimer interface of the Fe protein involves many interactions between the side chains of the conserved residues.

CHANGES TO THE METAL CLUSTERS UPON COMPLEX FORMATION

Nitrogenase complex formation causes redox potential changes to the metal clusters of the Fe protein and the MoFe protein. Studies of the L127Δ Fe protein showed that upon the L127Δ Fe protein-MoFe protein complex formation, the midpoint potential of the $[4Fe\text{-}4S]^{2+/+}$ couple of the Fe protein decreases from -420 mV to -620 mV, and the midpoint potential of the $P^{2+/N}$ couple changes by -80 mV (Lanzilotta & Seefeldt, 1997b). Also, significant decreases in the redox potentials of the [4Fe-4S] cluster and the P cluster have been observed upon the wild-type nitrogenase complex formation in the presence of AlF_4^- and ADP (Spee et al., 1998), resulting in an increase in the driving force for electron transfer from the Fe protein to the MoFe protein. Similar increases in the driving force for electron transfer upon protein complex

formation also occur in other protein complexes such as photosystem I with plastocyanin, and methylamine dehydrogenase with amicyanin (Chen et al., 1992; Drepper et al., 1996).

MECHANISM OF NUCLEOTIDE HYDROLYSIS

After the wild-type Fe protein with MgATP bound forms a complex with the MoFe protein, a single electron is transferred from the Fe protein to the MoFe protein, an event that is coupled to MgATP hydrolysis in the Fe protein (Burgess & Lowe, 1996; Seefeldt, 1997). The Fe protein cannot hydrolyze MgATP in the absence of the MoFe protein. Given that the distance between the Fe protein-MoFe protein binding surface and the nucleotide binding sites in the Fe protein is 17 Å (Schindelin et al., 1997), the MoFe protein cannot contribute critical catalytic groups for MgATP hydrolysis. Thus, it is reasonable that the binding of the Fe protein to the MoFe protein induces additional conformational changes in the Fe protein that reposition residues in the Fe protein to activate MgATP hydrolysis. Using MgATPγS with two of the γ-phosphate oxygen atoms labeled with ^{18}O and ^{17}O, it has been shown that MgATP hydrolysis occurs in the Fe protein by direct nucleophilic attack without covalent linking of the intermediate to the enzyme (Mortenson et al., 1985). Further studies on MgATP hydrolysis using radiolabeled ATP showed that it is the P(β)O-P(γ) bond that is broken during MgATP

hydrolysis (McKenna et al., 1989). This implies that during MgATP hydrolysis, a nucleophile, probably an activated water, attacks the phosphorus of the γ-phosphate, resulting in the formation of MgADP and free phosphate.

Mechanisms for nucleotide hydrolysis have been proposed for several nucleotide switch proteins such as G proteins, p21ras, and elongation factor-Tu. Comparison of the high-resolution X-ray structures of the $G_{i\alpha 1}$ with GTPγS bound and the $G_{i\alpha 1}$ with GDP• AlF_4^- bound revealed roles for Gln 204 and Arg 178 in GTP hydrolysis (Coleman et al., 1994). In the structure of GTPγS•$G_{i\alpha 1}$, there are no direct contacts between Gln 204 and solvent molecules and no interactions between the GTPγS and Arg 178. In contrast, significant reorientations of these two residues have been observed in the structure of $G_{i\alpha 1}$•GDP•AlF_4^-. The side chain of Arg 178 forms hydrogen bonds with two F atoms of AlF_4^-, which would make Arg 178 capable of stabilizing the negative charge developed at the pentacoordinate phosphate intermediate. The side chain of Gln 204 is hydrogen bonded with a water molecule, which is 1.9 Å away from the Al of AlF_4^-. This Gln may abstract a proton from the water, thus activating the water for GTP hydrolysis.

The crystal structure of $G_{t\alpha}$•GDP•AlF_4^- also implicates the side chain of a glutamine residue (Gln 200) in activating the attacking water. This residue would also

play a role in stabilizing the pentavalent phosphate intermediate. Arg 174 of the $G_{t\alpha}$ is suggested to facilitate product formation (Sondek et al., 1994).

The mechanism of GTP hydrolysis of p21ras is of particular interest as the rate of GTP hydrolysis in p21ras controls cell growth and differentiation in eukaryotes. Gln 61 in p21ras has been suggested to play a role in water activation for GTP hydrolysis, and mutation of this residue results in a decrease in the rate of GTP hydrolysis by more than four orders of magnitude (IS, 1988). The binding of GAP (GTPase activating protein) to p21ras greatly enhances the GTP hydrolysis rate of p21ras (Bollag & McCormick, 1991). Examination of the crystal structure of the p21ras•GAP•GDP•AlF_4^- complex supported the proposed role of Gln 61 in p21ras (Scheffzek et al., 1997). It also showed that the side chain of Arg 789 of GAP-334 moves into the active site of p21ras and participates in GTP hydrolysis by stabilizing the negative charge during formation of the intermediate. Another reason why GAP increases the GTPase activity of p21ras is that GAP functions in stabilizing the switch II region of p21ras, thus helping reorient Gln 61 for GTP hydrolysis.

Analysis of elongation factor Tu (EF-Tu) also provides some insight into the mechanism of nucleotide hydrolysis. Substitution of His 84 by Gly in EF-Tu does not change the affinity of the altered protein for GDP or GTP but does reduce the GTPase activity of EF-Tu to 5%. His 84 in EF-Tu may act as a general base to abstract a proton

from the attacking water (Cool & Parmeggiani, 1991). The crystal structure of EF-Tu•GppNHp revealed that there is no water molecule interacting with the side chain of His 84. However, it is proposed that binding of the ribosome to the effector region of EF-Tu may cause a conformational change in EF-Tu that will result in a rotation of the C_α-C_β bond of His 84, bringing it close to a water molecule that can be activated (Berchtold et al., 1993). Another residue in EF-Tu, Arg 58, is thought to stabilize the transition state during GTP hydrolysis (Limmer et al., 1992).

Taken together the mechanisms for nucleotide hydrolysis in these proteins, two conclusions can be drawn. First, a specific residue in the protein can function as a general base to activate the water, and some residues serve to stabilize the transition state of nucleotide hydrolysis. Second, the reorientations of these catalytic residues have been observed during the transition state formation.

The X-ray crystal structure of the AlF_4^- stabilized nitrogenase complex shows that two residues, Asp129 and Lys10, which do not cross the subunit interface in the crystal structure of the Fe protein alone (Schlessman et al., 1998), move across the subunit interface and interact with the bound nucleotide in the opposite subunit (Schindelin et al., 1997). Asp 129 interacts with the AlF_4^-, probably through a bound water molecule, and Asp 129 has been proposed to activate a water molecule for attacking the γ-phosphate. Changing Asp 129 to Glu resulted in an altered Fe protein,

which could bind to the MoFe protein, but could not hydrolyze MgATP (Lanzilotta et al., 1995b). The terminal NH_2 group of the Lys 10 side chain is close to the F atoms of AlF_4^- and to a terminal oxygen of the β- phosphate. Therefore, in homology to the proposed mechanisms for other nucleotide switch proteins, Lys 10 may play a role in MgATP hydrolysis by stabilizing the intermediate state or facilitating the product formation.

ELECTRON TRANSFER AND MGATP HYDROLYSIS UPON NITROGENASE COMPLEX FORMATION

Characterization of the wild-type and altered Fe proteins showed that MgATP hydrolysis can occur independent of electron transfer. After the Fe protein associates with the MoFe protein, MgATP can be hydrolyzed. In the absence of reductant, such as dithionite, the oxidized wild-type Fe protein can associate with the MoFe protein and hydrolyze MgATP without electron transfer (Thorneley et al., 1991). It has also been shown that in the presence of dithionite, the D39N Fe protein-MoFe protein complex can hydrolyze MgATP continuously without detection of substrates reduction (Lanzilotta et al., 1997a). Similar results have been observed for other nitrogenase complexes, such as the L127Δ Fe protein-MoFe protein complex (Ryle, M. J., unpublished results) and the *C. pasteurianum* Fe protein-*A. vinelandii* MoFe protein

complex (Larsen et al., 1995; Chan et al., 1999b).

Electron transfer can also occur without MgATP hydrolysis. Early studies showed that the Fe protein from *C. pasteurianum* can form a tight complex with the MoFe protein from *A. vinelandii* (Chan et al., 1999b; Emerich et al., 1978). Recently, it was found that the *C. pasteurianum* Fe protein can transfer a single electron to the MoFe protein without MgATP present and that the addition of MgATP accelerates the rate of electron transfer between the *C. pasteurianum* Fe protein and the *A. vinelandii* MoFe protein from 0.007 s^{-1} to approximate 100 s^{-1} (Chan et al., 1999b). An altered Fe protein that has Leu 127 deleted in the switch II region can also transfer an electron to the MoFe protein in the absence of MgATP. Studies of the electron transfer within the L127Δ Fe protein-MoFe protein complex indicated that MgATP increases the electron transfer rate from 0.2 s^{-1} to 5 s^{-1} (Lanzilotta et al., 1996a).

As electron transfer from the Fe protein to the MoFe protein is coupled to MgATP hydrolysis in the wild-type nitrogenase (Burgess & Lowe, 1996; Eady et al., 1978), understanding the role of ATP hydrolysis in electron transfer is important to understanding the nitrogenase mechanism. The energy of MgATP binding and hydrolysis could serve two functions in electron transfer. MgATP within the nitrogenase complex may accelerate the rate of electron transfer from the Fe protein to the MoFe protein. MgATP could also function in sending subsequent electrons from

the [4Fe-4S] cluster of the Fe protein to the P-cluster of the MoFe protein. This is based on the observation that a second electron could not be sent from the reduced L127Δ Fe protein to the MoFe protein within the nitrogenase complex (Lanzilotta et al., 1996a).

DISSOCIATION OF THE FE PROTEIN FROM THE MOFE PROTEIN

MgATP hydrolysis and phosphate release will leave the Fe protein in the MgADP bound state, which is believed to initiate the dissociation of the Fe protein from the MoFe protein (Lanzilotta et al., 1997a). Characterization of the D39N Fe protein revealed this altered Fe protein cannot undergo the same conformational changes as the wild-type Fe protein upon MgADP binding, which may account for its inability to dissociate from the MoFe protein after electron transfer.

The dissociation of the Fe protein from the MoFe protein is believed to be the rate-limiting step that controls the overall reaction rate of nitrogenase catalysis (Hageman & Burris, 1978). The D39N Fe protein is unable to reduce substrates (Lanzilotta et al., 1997a), suggesting that the dissociation of the Fe protein from the MoFe protein is a necessary step in nitrogenase catalysis. Other non-dissociating nitrogenase complexes, such as the L127Δ Fe protein-MoFe protein complex, and the *C. pasteurianum* Fe protein-*A. vinelandi*i MoFe protein complex, are also found to be

unable to reduce substrates (Ryle & Seefeldt, 1996b; Chan et al., 1999b).

However, there is also evidence arguing that the dissociation step may not be required for nitrogenase catalysis. Studies on the L127Δ Fe protein showed that the oxidized L127Δ Fe protein can be reduced within the nitrogenase complex by low potential electron transfer agents such as flavodoxin, which is believed to be the physiological reductant of the Fe protein (Lanzilotta et al., 1996a). In addition, experimental data support that nucleotide exchange in the Fe protein can occur while the Fe protein is still complexed with the MoFe protein (Lanzilotta et al., 1997a). Given that both reduction of the oxidized Fe protein and nucleotide exchange can occur within the Fe protein-MoFe protein complex, the dissociation of the Fe protein from the MoFe protein may not be a required step in nitrogenase catalysis. In support of this, modelling and analysis of kinetic data of the Fe protein cycle have suggested that the dissociation is not a necessary part of catalysis (Duyvis et al., 1998). The details of the actual steps of physiological nitrogenase catalysis still need to be investigated.

THE P-CLUSTER OF THE MOFE PROTEIN

The MoFe protein contains two kinds of metal clusters. The P-cluster is the [7Fe-8S] cluster bridging the α and β subunits of the MoFe protein (Kim & Rees, 1992). The P^N state of the P-cluster is EPR silent. The P^{1+} shows a perpendicular mode

EPR spectrum. The P^{2+} state exhibits a parallel mode EPR spectrum. The P-cluster is thought to mediate electron transfer from the [4Fe-4S] cluster of the Fe protein to the FeMo cofactor of the MoFe protein based on the position of the P-cluster in the MoFe protein as well as on experimental evidence. A protein containing homocitrate-less FeMo cofactor, called MoFe cluster, is not able to reduce substrate but can still undergo the P-cluster redox changes (Ma et al., 1996). In a separate study, when the reduced Fe protein with MgATP was mixed with the MoFe protein with the P-clusters in the P^{2+} state, the Fe protein EPR signal disappeared as a result of the oxidation of the [4Fe-4S] cluster cocurrent with the disappearance of the P^{2+} EPR signal and the appearance of the P^{1+} EPR signal, indicating electron transfer from the Fe protein to the P-cluster (Lanzilotta & Seefeldt, 1996b). Recently, an altered MoFe protein that has Ser 188 in the β subunit substituted by Cys, exhibited EPR spectral changes reflecting redox changes in the P-cluster during nitrogenase turnover (Chan et al., 1999a).

THE FEMO COFACTOR OF THE MOFE PROTEIN

The FeMo cofactor is the site where substrate reduction occurs. In the as-isolated MoFe protein, the FeMo cofactor exists in the S=3/2 state, demonstrating an EPR signal with g-values of 2.0, 3.7, and 4.3. Nitrogenase can reduce a wide variety of substrates such as nitrogen, protons, acetylene, and cyanide. Nitrogen and protons are

physiological substrates of nitrogenase. A lot of work has been done in order to understand the mechanism regarding the binding of substrates and reduction at the FeMo cofactor and understanding this mechanism is central to understanding the nitrogenase catalysis (Burgess & Lowe, 1996).

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CHAPTER 2

CHARACTERIZATION OF A HETERODIMERIC FE PROTEIN: DEFINING ROLES FOR ATP IN NITROGENASE CATALYSIS

ABSTRACT: Two ATP molecules bind to the homodimeric Fe protein component of nitrogenase, one to each subunit, and are hydrolyzed to ADP and P_i in a reaction coupled to the transfer of one electron into the MoFe protein component. As an approach to assess the contributions of individual ATP binding sites, a heterodimeric Fe protein was produced that has an Asn substituted for residue 39 in the ATP binding domain in one subunit, while the normal Asp^{39} residue within the complementary subunit remains unchanged. Three forms of the Fe protein (wild-type homodimeric Fe protein [Asp^{39}/Asp^{39}], altered homodimeric Fe protein [Asn^{39}/Asn^{39}], and heterodimeric Fe protein [Asp^{39}/Asn^{39}]) were compared on the basis of the biochemical and biophysical changes elicited by nucleotide binding. Among those features examined were the MgATP- and MgADP-induced protein conformational changes that are manifested by the susceptibility of the [4Fe-4S] cluster to chelation and by alterations in the EPR, CD, and midpoint potential of the [4Fe-4S] cluster. The results support a model where changes in the [4Fe-4S] cluster caused by nucleotide binding are the result of additive conformational changes contributed by the individual subunits. The

heterodimeric Fe protein did not support substrate reduction activity, but did hydrolyze MgATP and showed MgATP-dependent primary electron transfer to the MoFe protein. These results support a model where each MgATP site contributes to the rate acceleration of primary electron transfer, but both MgATP sites must be functioning properly for substrate reduction. Like the altered homodimeric [Asn^{39}/Asn^{39}] Fe protein, the heterodimeric [Asp^{39}/Asn^{39}] Fe protein was found to form a high affinity complex with the MoFe protein, revealing that alteration on one subunit is sufficient to create a tight complex.

INTRODUCTION

Molybdenum-dependent nitrogenase is composed of two component proteins called the MoFe protein and the Fe protein. The MoFe protein is a 240 kDa $\alpha_2\beta_2$ tetramer, with each $\alpha\beta$ dimeric unit containing one FeMo cofactor [Mo-8Fe-9S-homocitrate] and one [8Fe-7S] (P-) cluster (1). FeMo-cofactor provides the site of substrate reduction (2), while the P-cluster is thought to provide an intermediate electron transfer site between the Fe protein and FeMo cofactor (3-6). The Fe protein is a 64 kDa homodimer that has one nucleotide binding site on each subunit and a single [4Fe-4S] cluster bridged between the two subunits (7). During catalysis, the Fe protein with two bound MgATP molecules associates with the MoFe protein. This association

activates the hydrolysis of the two MgATP molecules, an event that is coupled to the transfer of a single electron from the Fe protein [4Fe-4S] cluster into the MoFe protein (8,9). The oxidized Fe protein, with two bound ADP molecules, then dissociates from the MoFe protein in a step that is believed to be rate-limiting for nitrogenase catalysis (10). This process is repeated until sufficient electrons have accumulated within the MoFe protein to permit substrate binding and reduction (11).

How MgATP binding and hydrolysis are coupled to intercomponent electron transfer and substrate reduction is a poorly defined aspect of the nitrogenase mechanism. The Fe protein is known to bind two nucleotides (either MgATP or MgADP) with the two binding events showing strong positive cooperativity (12). The binding of two MgADP or MgATP molecules induces protein conformational changes that have been detected as changes in the overall shape of the Fe protein (13) and as changes in the electronic and redox properties of the [4Fe-4S] cluster (14,15). These nucleotide-induced protein conformational changes are thought to have an important role in the nitrogenase mechanism.

One approach that has been used to probe the nitrogenase catalytic mechanism has involved placing amino acid substitutions within the nucleotide binding sites located within the Fe protein (16-21). One amino acid substitution in the homodimeric Fe protein that has proven informative is the substitution of residue Asp^{39} by Asn^{39} (20).

Inspection of the crystallographic structure of the Fe protein (7) indicates that the carboxylate of Asp^{39} interacts with bound nucleotide through the Mg^{++} cation. Substitution of Asp^{39} by Asn does not appear to significantly alter the binding of the nucleotides to the Fe protein, but it does alter the coupling of this binding to downstream events (20). For example, the MgATP and MgADP induced protein conformational changes elicited by nucleotide binding are impacted for the altered protein. In addition, the altered Fe protein shows only limited MgATP hydrolysis when combined with the MoFe protein and no substrate reduction can be detected. The altered Fe protein is capable of very slow electron transfer to the MoFe protein, but following electron transfer, the altered Fe protein does not effectively dissociate from the MoFe protein.

Because the Fe protein is a homodimer with each subunit coded for by a single *nif*H gene (22), any mutation results in production of an altered homodimeric Fe protein that carries an amino acid substitution on each subunit. In the present work, we have developed genetic and biochemical strategies for the production and purification of a heterodimeric Fe protein for which one subunit retains the normal Asp^{39} residue and the corresponding position in the other subunit is substituted by Asn^{39}. In this way it was possible to evaluate the contributions provided by each subunit of the Fe protein to events initiated and controlled by nucleotide binding and hydrolysis.

EXPERIMENTAL PROCEDURES

Strain Constructions. Refer to the paper (Chan, J.C., et al., (2000) *Biochemistry*, *39*, 7222).

Protein Expression and Purification. Wild-type Fe and MoFe proteins were expressed in *A. vinelandii* cells and purified as previously described (17,23) with specific activities > 1800 nmol C_2H_2 reduced/min/mg. Fe proteins were expressed from the appropriate strain using the urea/N_2-derepression protocol (17). Fe proteins were purified to apparent homogeneity by a combination of ion exchange and gel filtration chromatography as described (17). His-tagged Fe proteins were further purified using a Zn-affinity chromatography system (24), with step gradient elution with buffer containing either 75 or 250 mM imidazole. Protein concentrations were determined by a modified biuret method using bovine serum albumin as the standard (25). Fe protein concentrations were also determined from the visible absorption spectrum using an absorption coefficient for the oxidized protein of 13.3 $mM^{-1} \cdot cm^{-1}$ at 400 nm (26). All manipulations of proteins were conducted in the absence of oxygen in sealed serum vials under an argon atmosphere or inside of an anaerobic glove box (Vacuum Atmospheres, Hawthorne, CA) with an argon gas atmosphere.

Protein Characterization. SDS-PAGE was done as previously described (27) with Coomassie blue staining. The molecular mass of each Fe protein subunit was

determined by matrix-assisted laser desorption time-of-flight mass spectrometry at the Biotechnology Core Facility of Utah State University. A 1 μL portion of protein solution (diluted in water with 0.1% trifluoroacetic acid) was mixed with 1.5 μL of matrix solution (a saturated solution of alpha-cyano-4-hydroxycinnamic acid in a 40% acetonitrile, 0.1 % trifluoroacetic acid solution in water) and allowed to dry on a target. Analysis was done with a TofSpec mass spectrometer (Micromass, Inc., Beverly, MA) with ionization using a nitrogen laser. The spectrometer was run in the linear mode with 20 kV acceleration voltage and in the positive ion mode. Both internal and external calibration were used to establish the mass range, with cytochrome c as the standard. N-terminal amino acid sequences were determined at the Protein and Nucleic Acid Shared Facility at the Medical College of Wisconsin, Milwaukee.

Activities. Proton and acetylene reduction activities were determined as previously described at 30°C using a MgATP regenerating system (28). MgATP hydrolysis rates were quantified following separation of ATP and ADP by high performance liquid chromatography (HPLC) with a Supelcosil LC-18 column (4.6 mm x 25 cm; Supelco, Bellefonte, PA), a mobile phase of 100 mM KH_2PO_4 buffer, pH 6.0, containing 14% (v/v) methanol and 4 mM tetrabutylammonium hydrogen sulfate, and flow rates of 1.3-2.0 mL • min^{-1} (29). Nucleotides were detected with a continuous

flow UV-visible detector and a molar absorption coefficient of 15.4 $mM^{-1} \cdot cm^{-1}$ at 259 nm for MgADP (30).

The ability of altered, inactive Fe proteins to bind to the MoFe protein was tested by a competition assay described earlier (21). Increasing quantities of the altered Fe protein (from 0 to 256 μg) were added to fixed concentrations of wild-type Fe protein (128 μg or 2 nmol) and MoFe protein (256 μg or 1 nmol). The percent of acetylene reduction activity remaining was plotted versus the ratio of altered Fe protein to wild-type Fe protein, and the data were fit to the Hill equation (eqn 1)

$$\text{percentage activity} = V_{max} - (V_{max}[S]^n)/(K_m + [S]^n) \qquad \text{eqn 1}$$

where V_{max} is 100%, [S] is the concentration of active Fe protein, K_m is the Michaelis constant, and n is the cooperativity factor.

Spectroscopic Methods. X-band (9.64 GHz) EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a dual mode cavity and an Oxford ESR 900 liquid helium cryostat. All other parameters are noted in the figure legends.

Potentiometric redox titrations were performed essentially as previously described (19). Circular dichroism spectra were done on the indigo disulfonate oxidized Fe proteins essentially as previously described (31).

Fe Chelation. The chelation of iron from Fe proteins was followed spectrophotometrically by the formation of the Fe^{2+}-bathophenanthroline disulfonate

(BPS) complex, which was taken to have an absorption coefficient of 22.14 mM^{-1} • cm^{-1} at 534 nm (32). The Fe chelation assays were performed in 2 mL volume cuvettes with a 1 cm pathlength and fitted with serum stoppers. To each cuvette, 950 μL of a solution containing 1.0 mM BPS in 50 mM Tris buffer, pH 8.0, was added and deoxygenated by bubbling with argon for 6 min. Dithionite, ATP, and $MgCl_2$ were added to final concentrations of 2 mM, 1 mM, and 2 mM, respectively, and reactions were initiated by the addition of 4 mg (0.06 μmol) of Fe protein.

Stopped-Flow Spectroscopy. Absorbance changes resulting from the oxidation or reduction of the [4Fe-4S] cluster of the Fe protein were monitored with a Hi-Tech SF61 stopped-flow spectrophotometer equipped with data acquisition and curve fitting software (Salibury, Wilts, U.K.). The SHU-61 sample-handling unit was kept inside an anaerobic chamber with a gas atmosphere of 95% N_2 and 5% H_2 with less than 1 ppm oxygen. Reactants were thermostatted to $23.0 \pm 0.5^{\circ}$ C by means of a Techne C-85D circulator (Techne Ltd., Duxford, Cambridge, U.K.) attached to a FC-200 flow cooler (33). Data were collected at 430 nm. The reaction was initiated by rapid mixing of a solution containing 80 μM Fe protein and 10 mM dithionite in 50 mM HEPES buffer, pH 7.4, with a solution containing 20 μM MoFe protein, 10 mM dithionite, and 5 mM MgATP in 50 mM HEPES buffer, pH 7.4.

RESULTS

Construction and Purification of a Heterodimeric Fe Protein. Refer to the paper (Chan, J.C., et al., (2000) *Biochemistry*, *39*, 7224).

Identification of the Heterodimeric Fe Protein. The identity of the various Fe proteins used in the present work was confirmed by SDS-PAGE, mass spectrometry, and N-terminal protein sequence analysis. SDS-PAGE analysis of the heterodimeric Fe proteins revealed two protein bands on the SDS-gel. The slower migrating band (predicted to be a subunit with a His-tag) migrated identically with the one band observed for the homodimeric [Asp^{39}/Asp^{39}] Fe protein for which both subunits carry a His-tag. The faster migrating band (predicted to be a subunit without a His-tag) migrated identically with wild-type Fe protein and homodimeric [Asn^{39}/Asn^{39}] Fe proteins, neither of which carries a His-tag on either subunit (data not shown).

The molecular mass of the subunits of the various Fe proteins was determined by mass spectrometry, and the results are shown in Table 2-1. These results are all in line with predictions based on the known primary sequences of the individual subunits for each construct (22). The heterodimeric Fe protein showed two parent peaks in the mass spectrum, each with nearly identical intensity. One of these peaks shows a mass consistent with the presence of a His-tag whereas the other peak corresponds to the mass of a subunit that does not carry a His-tag (Table 2-1). Finally, the N-terminal

Table 2-1: N-Terminal Amino Acid Sequences and Mass Spectral Data for Fe Proteins

Fe protein	subunit mass (Da)		
subunit composition	sequence[a]	mass spectrometry	N-terminal sequence
[Asp^{39}/Asp^{39}][b]	31,365	31,317	AMRQCAIYGKGG[a]
[Asp^{39}/Asp^{39}][c]	32,587	32,565	MHHHHHHMAMR
[Asp^{39}/Asn^{39}][d]	31,365	31,413	AMRQX[e]AIYGKGG
	32,587	32,547	MHHHHHHMAMR

[a] Sequence predicted from the DNA sequence of the *nifH* gene (22). [b]Fe protein produced by the wild-type strain. Does not carry a His-tag on either subunit. [c]Third Fe protein fraction eluted using the immobilized metal affinity chromatography technique. This protein is predicted to have an N-terminal His-tag on both subunits. [d]Second Fe protein fraction eluted using the immobilized metal affinity chromatography technique. This protein is predicted to have one subunit that carries a His-tag and one subunit that does not contain a His-tag. [e]No single amino acid was detected at this position.

sequence was determined for the heterodimeric [Asp^{39}/Asn^{39}] Fe protein and compared to the N-terminal sequences of the wild-type Fe protein and an Fe protein that carries a His-tag on both subunits of an otherwise wild-type protein (Table 2-1).

Characterization of His-Tagged Fe Proteins That Do Not Carry Amino Acid Substitutions. It was first necessary to determine the effect of N-terminal His-tags on Fe protein catalytic function. Fe proteins carrying a His-tag on either one or both of the subunits, but no other alterations within the Fe protein coding sequence, were observed to support high rates of proton and acetylene reduction activity (Table 2-2). MgATP hydrolysis rates were also similar to those supported by the non-tagged Fe protein, and ATP/e^- ratios were nearly identical to those of non-tagged Fe protein. The Fe protein to MoFe protein ratio required for optimal activity was similar for all Fe proteins examined. In addition, the nucleotide-induced conformational changes observed for His-tagged Fe proteins that are otherwise unaltered were identical to those of the non-tagged wild-type Fe protein as assessed by EPR, CD, midpoint potential determinations, Fe chelation rates, and primary electron transfer rates (described below). These results demonstrate that the presence of an N-terminal His-tag on one or both subunits of the Fe protein does not prevent its proper functioning in catalysis. Wild-type Fe protein in the following sections refers to Fe protein with either one or two His-tags.

Table 2-2: Activities of Fe Proteins

Fe protein subunit composition	specific activity (nmol product • min^{-1} • mg Fe $protein^{-1}$)			
	C_2H_4	H_2	MgADP	ATP/e-
[Asp^{39}/Asp^{39}][a]	1810 ± 20	1960 ± 40	4640 ± 580	2.6 ± 0.4
[Asp^{39}/Asp^{39}][b]	1470 ± 20	1460 ± 60	3320 ± 230	3.2 ± 0.5
[Asn^{39}/Asn^{39}][c]	ND[e]	ND	14.0 ± 0.3	--
[Asp^{39}/Asn^{39}][d]	ND	ND	280 ± 20	--

[a]Fe protein produced by the wild-type strain. Does not contain a His-tag on either subunit. [b]Fe protein purified from strain DJ1298. One subunit contains an N-terminal His-tag, and the other subunit does not contain a His-tag. [c]Heterodimeric [Asn^{39}/Asn^{39}] Fe protein. Does not contain a His-tag on either subunit. [d]Fe protein purified from DJ1274. One subunit contains an N-terminal His-tag, and the other subunit does not contain a His-tag. [e]Not detected.

Activities of the Heterodimeric [Asp^{39}/Asn^{39}] Fe Protein. Fe protein that has the Asp^{39} residue position substituted by Asn^{39} within both subunits showed no substrate reduction activity and only trace MgATP hydrolysis activity as reported earlier [(20), Table 2-2]. Similarly, the heterodimeric [Asp^{39}/Asn^{39}] Fe protein did not exhibit detectable substrate reduction activities, although this altered Fe protein did show MgATP hydrolysis activity that was approximately 5% of the wild-type rate.

Nucleotide Interactions with the Heterodimeric Fe Protein. The wild-type Fe protein is known to bind two molecules of MgATP or MgADP (7), with the binding of either nucleotide resulting in characteristic changes in the lineshape of the EPR spectrum of the $[4Fe\text{-}4S]^{1+}$ cluster (14,34,35), the visible region CD spectrum (15,31), the chelation rate of Fe from the cluster (32,36), and the E_m for the $[4Fe\text{-}4S]^{2+/1+}$ cluster couple (14). Monitoring of these features was then used to assess changes in the coupling of nucleotide binding to protein conformational changes within the heterodimeric [Asp^{39}/Asn^{39}] Fe protein.

All of the Fe proteins studied in the present work showed identical resting state, rhombic EPR spectra in the absence of nucleotides, confirming that substitution of Asp^{39} within one or both Fe protein subunits does not alter the resting state of the [4Fe-4S] cluster. Addition of MgATP to the wild-type Fe protein (or to His-tagged versions of the wild-type Fe protein) results in a lineshape change to an axial signal ($g = 2.03$ and

1.92) (Figure 2-1). In contrast, an altered homodimeric [Asn^{39}/Asn^{39}] Fe protein shows little change in EPR spectrum upon addition of MgATP (g = 2.06, 1.94, and 1.86), indicating that the amino acid change has prevented MgATP coupled changes to the electronic properties of the [4Fe-4S] cluster. Addition of MgATP to the heterodimeric [Asp^{39}/Asn^{39}] Fe protein was observed to result in an EPR spectrum with g = 2.04, 1.93, and a poorly defined third g-value. This lineshape change appeared to be between the MgATP-bound wild-type Fe protein spectrum and the MgATP-bound form of the altered homodimeric [Asn^{39}/Asn^{39}] Fe protein spectrum, suggesting a partial conformational change resulting from MgATP binding to the heterodimeric Fe protein.

The visible region CD spectrum of the oxidized Fe protein reports conformational changes around the oxidized [4Fe-4S] cluster upon binding nucleotides (Figure 2-2). Earlier studies showed that the Fe protein alone, the Fe protein with bound MgATP, and the Fe protein with bound MgADP each exhibit a unique CD spectrum (31). Substituting Asp^{39} by Asn^{39} within both Fe protein subunits was previously found not to alter either the nucleotide-free or the MgATP-bound CD spectrum when compared to wild-type Fe protein (20). However, transition to the MgADP-bound spectrum was prevented in the case of the homodimeric [Asn^{39}/Asn^{39}] form of the Fe protein. The heterodimeric [Asp^{39}/Asn^{39}] Fe protein also exhibited normal nucleotide-free and MgATP-bound spectra, indicating that substituting Asp^{39} to

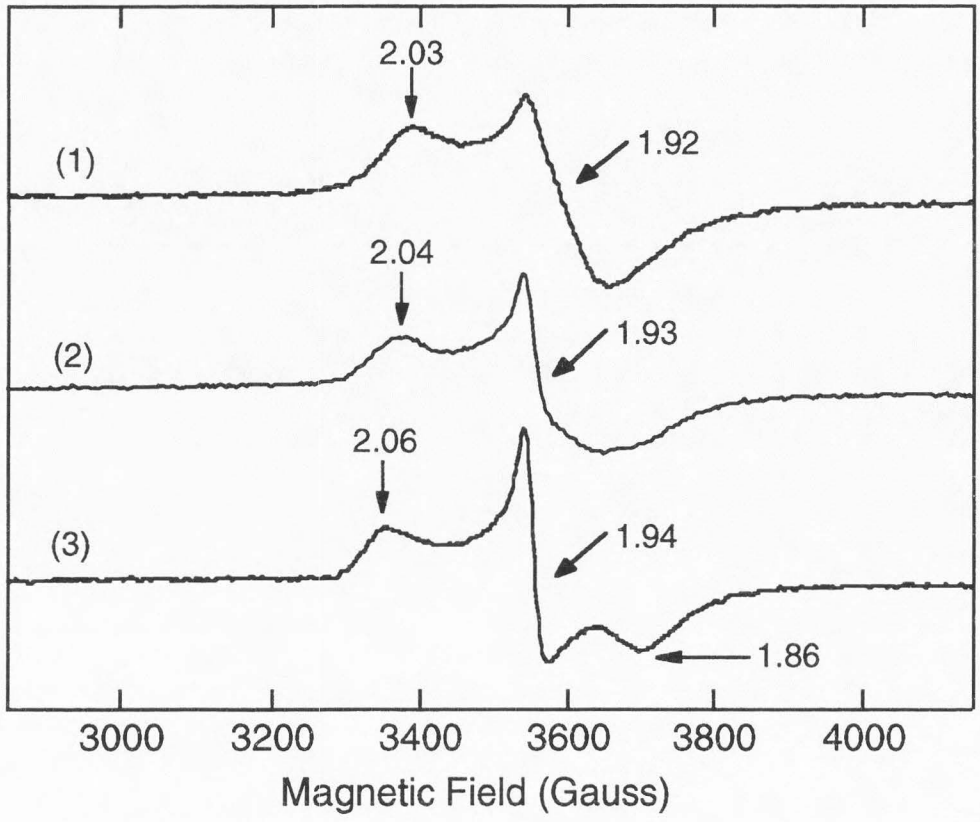

FIGURE 2-1: EPR spectra of Fe proteins. Perpendicular mode EPR spectra for: a wild-type homodimeric [Asp^{39}/Asp^{39}] Fe protein for which one subunit carries a His-tag (trace 1); a heterodimeric [Asp^{39}/Asn^{39}] Fe protein for which one subunit carries a His-tag (trace 2); and a homodimeric [Asn^{39}/Asn^{39}] Fe protein (trace 3). Spectra were recorded at 12K, with a microwave frequency of 9.64 GHz, a microwave power of 2 mW, a modulation frequency of 100 kHz, a modulation amplitude of 7.97 G, and a conversion time and time constant of 10.24 ms.

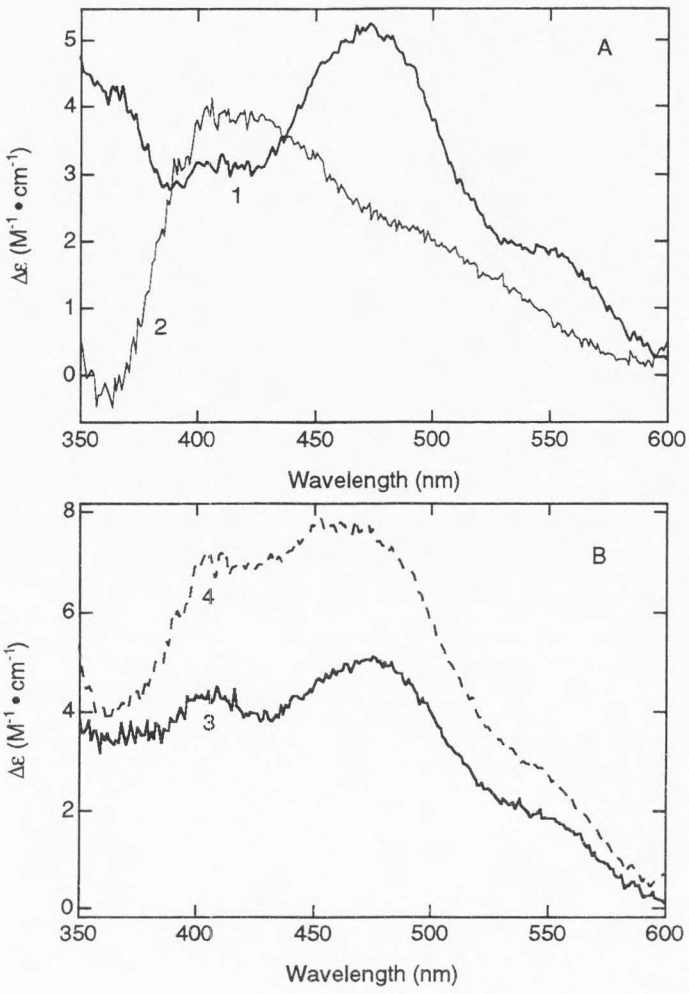

FIGURE 2-2: CD spectra of Fe proteins. The visible region CD spectra of IDS-oxidized Fe proteins were recorded as described under Experimental Procedures. In panel A the CD spectra for a wild-type homodimeric [Asp^{39}/Asp^{39}] Fe protein for which both subunits carry a His-tag (trace 1) and a homodimeric [Asn^{39}/Asn^{39}] Fe protein (trace 2) are shown. In panel B the spectrum of a heterodimeric [Asp^{39}/Asn^{39}] Fe protein for which one subunit carries a His-tag (trace 3) is shown. Trace 4 shows the additive spectrum of trace 1 + trace 2.

Asn^{39} for only one subunit does not perturb either the resting state of the protein or the MgATP-induced structural changes. However, the spectrum for the MgADP-bound form of the heterodimeric [Asp^{39}/Asn^{39}] Fe protein was similar to the additive spectrum of the wild-type Fe protein and the homodimeric [Asn^{39}/Asn^{39}] Fe protein spectra in their MgADP-bound forms, suggesting partial ADP induced conformational changes.

A sensitive way to monitor MgATP-induced protein conformational changes within the Fe protein is the rate of chelation of Fe from the [4Fe-4S] cluster by chelators such as bathophenanthroline disulfonate (BPS) (32,36). Earlier studies revealed that addition of MgATP, but not MgADP, to the Fe protein substantially increased the rate of formation of Fe^{2+}-BPS, which can be monitored by an absorbance increase (32). The substitution of Asp^{39} by Asn^{39} in both subunits was found to greatly diminish the rate of Fe chelation upon addition of MgATP (Figure 2-3). Substitution of Asp^{39} by Asn^{39} for only one Fe protein subunit resulted in a rate of Fe chelation that is between that observed for the wild-type Fe protein and the altered homodimeric [Asn^{39}/Asn^{39}] Fe protein.

The midpoint potential of the [4Fe-4S] $^{2+/1+}$ couple changes from -300 mV to -420 mV or -460 mV by the binding of MgATP or MgADP to the Fe protein, respectively (14,19). Substitution of Asp^{39} by Asn^{39} on both subunits of the Fe protein did not change the E_m (-300 mV) in the absence of nucleotides from that observed for

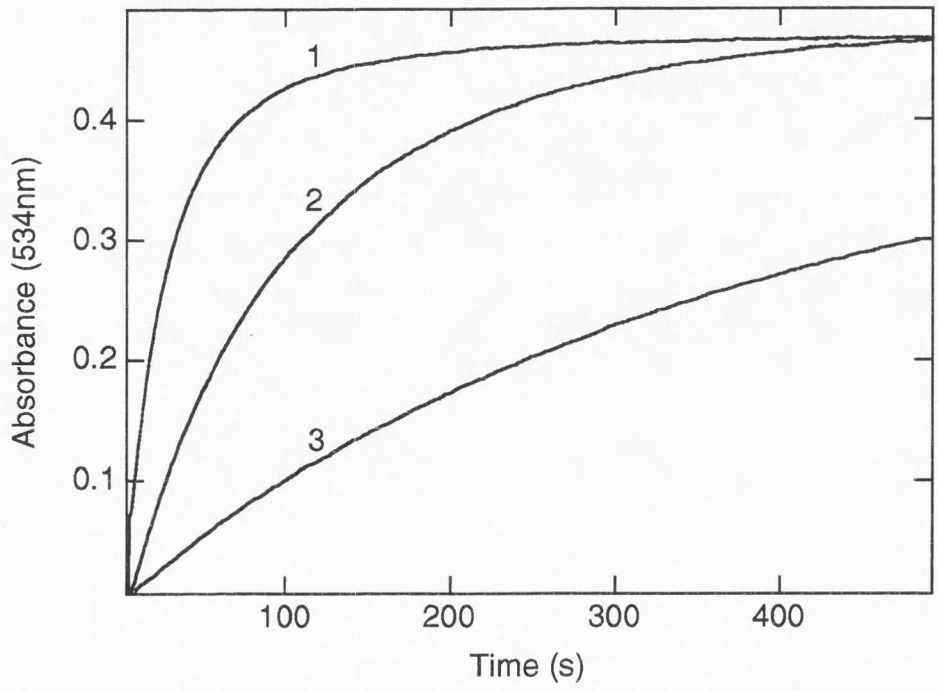

FIGURE 2-3: Time course for Fe chelation from Fe proteins. The time course for MgATP-dependent Fe chelation from Fe proteins was determined from the increase in absorbance at 534 nm from formation of Fe^{2+}-bathophenanthroline disulfonate (BPS) as described in the Experimental Procedures. The Fe proteins used were: a wild-type homodimeric [Asp^{39}/Asp^{39}] Fe protein for which one subunit carries a His-tag (trace 1); a heterodimeric [Asp^{39}/Asn^{39}] Fe protein for which one subunit carries a His-tag (trace 2); and a homodimeric [Asn^{39}/Asn^{39}] Fe protein (trace 3). Apparent first-order rate constants were 0.027, 0.009, and 0.003 s^{-1}, respectively.

the wild-type Fe protein. However, the addition of MgADP to the altered homodimeric [Asn^{39}/Asn^{39}] Fe protein did not lower the E_m. Examination of the heterodimeric [Asp^{39}/Asn^{39}] Fe protein revealed that MgADP changes the E_m from -300 mV to -390 mV, intermediate between those changes elicited for the wild-type and homodimeric [Asn^{39}/Asn^{39}] Fe proteins.

Interactions of a Heterodimeric Fe Protein with MoFe Protein. Substitution of Asp^{39} by Asn^{39} within both subunits of the Fe protein greatly diminishes the rate of MgATP-dependent electron transfer from the Fe protein to the MoFe protein (Table 2-3). More importantly, this altered Fe protein forms a non-dissociating complex with the MoFe protein following electron transfer. This situation contrasts with the wild-type Fe proteins, where dissociation from the MoFe protein occurs after electron transfer and MgATP hydrolysis. Examination of the heterodimeric Fe protein reveals that the rate of MgATP-dependent electron transfer to the MoFe protein is faster than for the altered homodimeric [Asn^{39}/Asn^{39}] Fe protein, but slower than for the wild-type Fe protein. This result indicates that each MgATP site contributes to the rate acceleration of electron transfer. Similar to the homodimeric [Asn^{39}/Asn^{39}] Fe protein, however, the heterodimeric [Asp^{39}/Asn^{39}] Fe protein inhibited wild-type Fe protein-MoFe protein complex formation (Figure 2-4), indicating that the heterodimeric Fe protein forms a tight complex with the MoFe protein.

Table 2-3: Primary Electron Transfer from Fe Proteins to MoFe Protein

Fe protein subunit composition	apparent first order rate constant (s^{-1})
[Asp^{39}/Asp^{39}][a]	118 ± 15[e]
[Asp^{39}/Asp^{39}][b]	120 ± 10
[Asn^{39}/Asn^{39}][c]	0.025 ± 0.009
[Asp^{39}/Asn^{39}][d]	2.2 ± 1.0

[a]Fe protein produced by the wild-type strain. Does not contain a His-tag on either subunit. [b]Fe protein purified from strain DJ1298. One subunit contains an N-terminal His-tag, and the other subunit does not contain a His-tag. [c]Altered Fe protein. Does not contain a His-tag on either subunit. [d]Fe protein purified from DJ1274. One subunit contains an N-terminal His-tag, and the other subunit does not contain a His-tag. [e]Standard deviations are from three independent measurements.

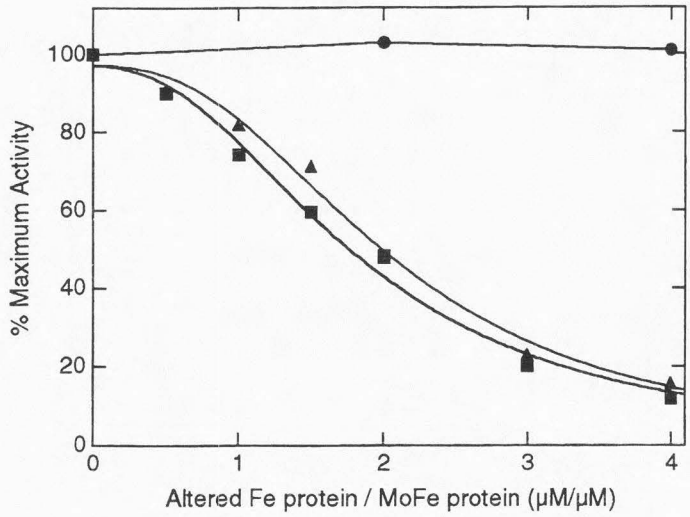

FIGURE 2-4: Inhibition of wild-type nitrogenase acetylene reduction activity by heterodimeric [Asp^{39}/Asn^{39}] Fe protein and homodimeric [Asn^{39}/Asn^{39}] Fe protein. Inhibition of wild-type nitrogenase acetylene reduction activity was performed as described in Experimental Procedures. 256 μg (1 nmol) of wild-type MoFe protein was added to each assay vial. Different amounts (from 32 to 256 μg, or 0.5 to 4 nmol) of homodimeric [Asn^{39}/Asn^{39}] Fe protein (■), heterodimeric [Asp^{39}/Asn^{39}] Fe protein for which one subunit carries a His-tag (▲), or bovine serum albumin (●) were added prior to initiation of the assay by the addition of 128 μg (2 nmol) of wild-type Fe protein. The percentage of the maximum activity observed in the absence of added inhibitor is plotted against the molar ratio of altered Fe protein to MoFe protein. The data were fit to the inverse Hill equation, where the cooperativity factor was found to be 2.5 for the heterodimeric [Asp^{39}/Asn^{39}] Fe and 2.3 for the homodimeric [Asn^{39}/Asn^{39}] Fe protein.

DI SCUSSION

ATP Interactions with Fe Protein. One of the open questions about the nitrogenase mechanism is how nucleotide binding and hydrolysis are coupled to conformational changes in the Fe protein. Extensive studies have been conducted to probe the protein conformational changes induced in the Fe protein upon binding nucleotides (8,9). Given the architecture of the Fe protein where nucleotides bind 15 Å away from the [4Fe-4S] cluster (7), protein conformational changes are the likely method for communication from the nucleotide binding site and the cluster (16,37). In all previous studies, these conformational changes were examined with nucleotides interacting with both subunits of the Fe protein (16-21,37-44). In the present study, using the heterodimeric Fe protein, it was possible to probe the contributions of nucleotide interactions from each subunit on the overall conformational changes imposed on the [4Fe-4S] cluster. The assessment of the nucleotide-induced protein conformations indicates that the heterodimeric [Asp^{39}/Asn^{39}] Fe protein undergoes conformational changes that are intermediate between those of the wild-type and altered homodimeric [Asn^{39}/Asn^{39}] Fe proteins. It therefore appears that the contributions to the protein conformational changes induced by each nucleotide binding are additive. This finding is supported by the earlier finding from titration calorimetry that suggested that the binding energy from two nucleotides is required to account for the change

induced in the E_m of the cluster (45).

ATP Roles in the Fe Protein-MoFe Protein Complex. Results from the current work also address roles played by two nucleotides within the Fe protein-MoFe protein complex. In the absence of MgATP, the Fe protein shows no detectable electron transfer to the MoFe protein. Upon addition of MgATP, primary electron transfer occurs with a rate constant of approximately 100 s^{-1} (46). For the altered homodimeric [Asn^{39}/Asn^{39}] Fe protein, the MgATP-dependent electron transfer rate is lowered by four orders of magnitude compared to that observed for the wild-type Fe protein. In contrast, the heterodimeric [Asp^{39}/Asn^{39}] Fe protein was observed to have an electron transfer rate of 2.2 s^{-1}, between the rates observed for the wild-type and [Asn^{39}/Asn^{39}] homodimeric Fe proteins. This suggests that the proper functioning of one subunit of the Fe protein with regard to MgATP provides partial stimulation of the rate of electron transfer. The full acceleration of the rate of electron transfer would be predicted to result from the additive effects of the two MgATP binding/hydrolysis reactions. The order of MgATP hydrolysis and electron transfer has not been clearly defined (47), so in the present work the rate acceleration could result from either MgATP binding or hydrolysis.

Finally, the present results reveal that alteration on only one subunit of the Fe protein is sufficient to activate the formation of a tight complex between the Fe protein

and the MoFe protein. Earlier it was found that the homodimeric [Asn^{39}/Asn^{39}] Fe protein formed a tight complex with the MoFe protein (20). In the present work, it is shown that the heterodimeric [Asp^{39}/Asn^{39}] Fe protein also forms a tight complex with the MoFe protein. Given that the docking interface on the MoFe protein is pseudosymmetric (48), it is possible that the heterodimeric Fe protein only forms a tight complex in a certain orientation with the MoFe protein.

In summary, characterization of a heterodimeric Fe protein for which the Asp^{39} residue within only one of the two Fe protein subunits was substituted by Asn^{39} has led to the following conclusions. The contributions from each subunit to the nucleotide-induced conformational changes that affect the biophysical features of the [4Fe-4S] cluster are additive. The acceleration of the rate of electron transfer from the Fe protein to the MoFe protein is the consquence of an additive effect resulting from the binding/hydrolysis of one MgATP to each Fe protein subunit. The formation of a tight Fe protein-MoFe protein complex can result from the modification of only one subunit of the Fe protein.

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CHAPTER 3

EVIDENCE THAT NITROGENASE FE PROTEIN-MOFE PROTEIN COMPLEX FORMATION ACTIVATES A ROLE FOR LYSINE 10 OF THE FE PROTEIN IN ATP HYDROLYSIS

ABSTRACT: Substrate reduction catalyzed by nitrogenase requires hydrolysis of MgATP. MgATP hydrolysis is coupled to electron transfer from the nitrogenase iron (Fe) protein to the molybdenum-iron (MoFe) protein and allows the Fe protein to dissociate from the MoFe protein. This work presents evidence that Lys 10 of the Fe protein plays a role in stimulating MgATP hydrolysis after the Fe protein-MoFe protein complex formation. Changing Lys 10 of the Fe protein to Arg resulted in an Fe protein (K10R) that hydrolyzed MgATP at a rate 3% that of the wild-type Fe protein. The altered K10R Fe protein could not support substrate reduction. The interactions of the K10R Fe protein with nucleotides appear to be similar to the wild-type Fe protein based on the affinity of the K10R Fe protein for nucleotides and conformational changes of the K10R Fe protein upon nucleotide binding. The significant decrease in the MgATP hydrolysis rate of the K10R Fe protein indicates a role of Lys 10 of the Fe protein in ATP hydrolysis after nitrogenase complex formation. Given that the K10R Fe protein dissociated from the MoFe protein at a rate similar to the wild-type Fe protein as

determined by stopped-flow spectrophotometry, the inability of the K10R Fe protein to reduce substrates probably was due to the very low rate of MgATP hydrolysis. This work supports the proposed role of Lys 10 of the Fe protein deduced from the crystal structure of the ADP•AlF_4^- stabilized nitrogenase complex showing that Lys 10 could stabilize the leaving phosphate of the MgATP during hydrolysis.

INTRODUCTION

Nitrogenase is the enzyme that catalyzes biological nitrogen fixation in a reaction requiring 16 MgATP for each N_2 reduced. The two component proteins of nitrogenase are the iron (Fe) protein and the molybdenum-iron (MoFe) protein. The Fe protein is a homodimer containing two nucleotide binding sites and one [4Fe-4S] cluster bridging the two identical subunits (1). The MoFe protein is a $\alpha_2\beta_2$ tetramer with two [8Fe-7S] clusters (P-cluster) and two molybdenum-iron-sulfur-homocitrate clusters (FeMo cofactor) (2). During nitrogenase catalysis, the Fe protein binds two MgATP molecules, which induce a conformational change in the Fe protein and allow the Fe protein to associate with the MoFe protein (3). After Fe protein-MoFe protein complex formation, an electron is transferred from the [4Fe-4S] cluster of the Fe protein to the MoFe protein in a reaction coupled to MgATP hydrolysis in the Fe protein (3, 4). Then the MgADP-bound Fe protein dissociates from the MoFe protein. The oxidized Fe

protein is reduced by cellular reductants and two MgADP in the Fe protein are exchanged by two MgATP (5). This cycle is repeated until enough electrons accumulate within the MoFe protein to reduce substrates (6).

Nucleotides play an important role in every step of nitrogenase catalysis. The binding of MgATP to the Fe protein lowers the midpoint potential of the [4Fe-4S] cluster by more than 100 mV (7, 8), which is probably important to facilitating electron transfer from the Fe protein to the MoFe protein. The binding of MgATP to the Fe protein also induces protein conformational changes which are necessary for the Fe protein to bind to the MoFe protein. MgATP hydrolysis is then coupled to the electron transfer event. Several studies have shown that MgATP hydrolysis is not absolutely required for electron transfer within the nitrogenase complex but functions in accelerating electron transfer (9-11). In these cases, the Fe protein forms a tight complex with the MoFe protein after electron transfer and no substrate reduction is detected. For the wild-type nitrogenase, MgATP hydrolysis is thought to stimulate the Fe protein to dissociate from the MoFe protein (12, 13) as the Fe protein with MgADP bound has reduced affinity for the MoFe protein.

The Fe protein cannot hydrolyze MgATP without the MoFe protein. Given the distance between the nucleotide binding site of the Fe protein and the Fe protein-MoFe protein docking surface, the MoFe protein does not appear to be directly involved in

nucleotide hydrolysis. It seems reasonable that Fe protein-MoFe protein complex formation induces a further conformational change within the Fe protein, which causes the reorientation of specific residues for the catalysis of MgATP hydrolysis. Figure 3-1 shows two molecular models of the Fe protein. The left model shows the A. *vinelandii* Fe protein in the absence of nucleotides, and the right model shows the A. *vinelandii* Fe protein in the ADP•AlF_4^- stabilized nitrogenase complex (14, 15). The crystal structure of the ADP•AlF_4^- stabilized nitrogenase complex (15) shows that two residues, Asp 129 and Lys 10, both cross the subunit interface and interact with the nucleotide in the other subunit. Earlier work on Asp 129 of the Fe protein suggested that this residue participates in MgATP hydrolysis by activating a water molecule to attack the γ-phosphate of MgATP (16). Lys 10 is located very close to the β-phosphate and the AlF_4^-, which is supposed to represent the position of the γ-phosphate of MgATP. It is proposed that Lys 10 can stabilize the leaving phosphate group, thus playing a role in nucleotide hydrolysis. To probe the role of this amino acid, site-directed mutagenesis was employed to change the codon for Lys 10 to the codon for Arg in the A. *vinelandii* Fe protein gene, *nif*H. This K10R Fe protein was completely characterized. The results of this work imply a role of Lys 10 of the Fe protein in MgATP hydrolysis. Lys 10 is not involved in nucleotide-induced conformational changes in the Fe protein prior to Fe protein-MoFe protein complex formation.

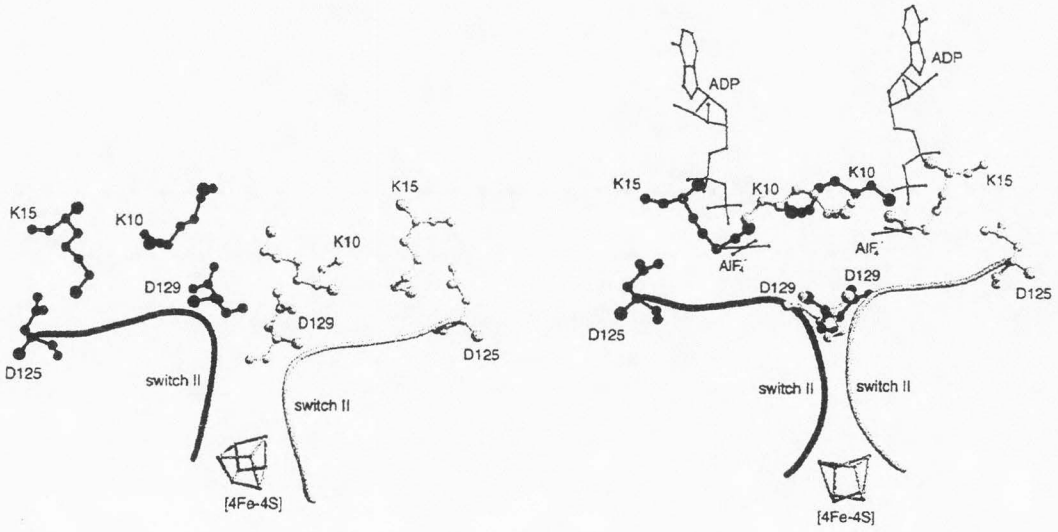

FIGURE 3-1: Molecular models of A. *vinelandii* Fe protein. The left picture shows the molecular model of A. *vinelandii* Fe protein in the absence of nucleotides (14). The right picture shows the molecular model of A. *vinelandii* Fe protein in the ADP · AlF_4^- - stabilized nitrogenase complex (15). The Lys 10, Lys 15, Asp 125, Asp 129 and the α-carbon trace of switch II (Asp 125 to Cys 132) from one subunit of the Fe protein are shown in dark color while the corresponding amino acids and switch II from the other subunit are shown in light color. The [4Fe-4S] cluster is also shown in both pictures. The position of ADP · AlF_4^- is shown in the right picture. All the models were generated using the program MOLSCRIPT (17) and Ratser3D (18).

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Expression, and Purification of Fe Proteins. Site-directed mutagenesis of the *nif*H gene that encodes the Fe protein subunit of the *Azotobacter vinelandii* nitrogenase, was carried out as previously described (19, 20). Expression and purification of the wild-type and K10R Fe proteins were also performed as previously described (21). All the Fe proteins were purified in 50 mM Tris buffer with 2 mM sodium dithionite, pH 8.0. The buffer used to purify the K10R Fe protein also includes 20% glycerol in order to stabilize the protein. Glycerol concentration was reduced to less than 5% by 50 mM Tris before freezing the protein in liquid nitrogen. Protein concentrations were determined by a modified biuret method using bovine serum albumin as the standard (22). SDS-polyacrylamide gel electropheresis with Coomassie blue staining was used to confirm that all the proteins were purified to homogeneity (23). Protein manipulations were performed either in a sealed serum vial with 2 mM dithionite and positive argon pressure or in a glove box with less than 1 ppm of oxygen.

Activity Assays. The acetylene and proton reduction rates, MgATP hydrolysis rates of the wild-type, and K10R Fe proteins were determined as previously described (24).

MgADP Binding to the Fe Protein. The affinities of the Fe proteins for MgADP

were determined using equilibrium column binding technique (25). Dithionite-reduced wild-type or K10R Fe protein (4 mg, initial protein concentration can vary) was loaded on the Sephadex G-25 column equilibrated with 50 mM Tris, pH 8.0, 2 mM dithionite and known concentration of MgADP from 0 to 1100 μM. The fraction collected before the protein comes out was analyzed for free nucleotide concentration by HPLC as previously described (20). The protein-containing fraction was split into two aliquots. One aliquot was analyzed for protein concentration using the modified biuret method. The other aliquot was analyzed for nucleotide concentration using the HPLC method. The data were fit to the Hill equation and the apparent dissociation constants were determined.

The Initial Rates of MgATP-Dependent Chelation of Fe^{2+} from Fe Proteins.

The chelation experiments were performed in a sealed cuvette containing 50 mM Tris, pH 8.0, 1 mM bathophenanthrolinesulfonic acid (BPS), 2 mM dithionite, and 0.4 mg (6.25 μM) wild-type or K10R Fe protein. The solution in the cuvette containing Tris buffer and BPS was first purged with O_2-free argon for 6 minutes before the addition of dithionite and protein. The reaction was initiated by the addition of certain concentration of MgATP from 0 to 2000 μM. The formation of the Fe^{2+}-BPS complex was monitored spectrophotometrically at 534nm by using an absorption coefficient of 22.14 mM^{-1} cm^{-1} (26). The initial rate of the chelation at different MgATP

concentration was calculated.

Circular Dichroism Spectra of Oxidized Wild-Type and K10R Fe Proteins. Circular dichroism spectra of oxidized Fe proteins were recorded as previously described (27). The wild-type or the K10R Fe protein (20 mg, initial protein concentration can vary) was desalted by passage through the Sephadex G-25 column equilibrated with 100 mM Tris, pH 8.0. Indigo disulfonate (IDS) of 25 mM (25 μL) was added to the Fe protein sample to oxidize the Fe protein. The IDS was removed from the Fe protein using a Dowex column equilibrated with 100 mM Tris, pH 8.0. The oxidized Fe protein was split equally into two 1 cm path length quartz cuvettes and then the Fe protein in each cuvette was diluted to 2 ml with 100 mM Tris, pH 8.0. Nucleotides were added to a final concentration of 1 mM from a 25 mM stock solution. All spectra were recorded by using an Aviv model 62DS spectropolarimeter and were baseline subtracted.

Stopped-Flow Spectrophotometry. Absorbance changes at 430 nm were monitored with a Hi-Tech SF61 stopped-flow spectrophotometer equipped with a data acquisition and curve fitting system (Salisbury, Wilts, UK) as previously described (9). Electron transfer rate from the Fe protein to the MoFe protein and rate of the dissociation of the IDS-oxidized Fe protein from the MoFe protein were determined as previously described (9).

RESULTS

The A. *vinelandii* cells expressing the K10R-altered proteins were unable to grow under nitrogen-fixing conditions but could grow if ammonia or urea is provided into the medium. In vitro analysis of the purified K10R Fe protein showed that the K10R Fe protein, unlike the wild-type Fe protein, could not support the acetylene reduction and the proton reduction when combined with the wild-type MoFe protein. The reductant-dependent MgATP hydrolysis rate of the K10R Fe protein was extremely low, approximately 3% the rate determined for the wild-type Fe protein.

Nucleotide Binding to the Wild-Type and K10R Fe Proteins. Since the K10R Fe protein did not support reduction of substrates, it is important to find out whether or not changing Lys 10 to Arg in the Fe protein affects the binding of nucleotides to the Fe protein. Figure 3-2 shows the graph where the ratio of the concentration of the bound MgADP to the Fe protein concentration was plotted against the free MgADP concentration. The data were fit to the Hill equation, $F_b=M_b[S]^n/(K_d+[S]^n)$, where F_b is the fraction of MgADP bound per Fe protein, M_b is the maximum number of MgADP bound per Fe protein, [S] is the free MgADP concentration, n is the cooperativity factor, and K_d is the apparent dissociation constant. The K10R protein was found to bind a maximum of 1.6 MgADP with the apparent dissociation rate (K_d) and the Hill cooperativity number similar to those obtained for the wild-type Fe protein.

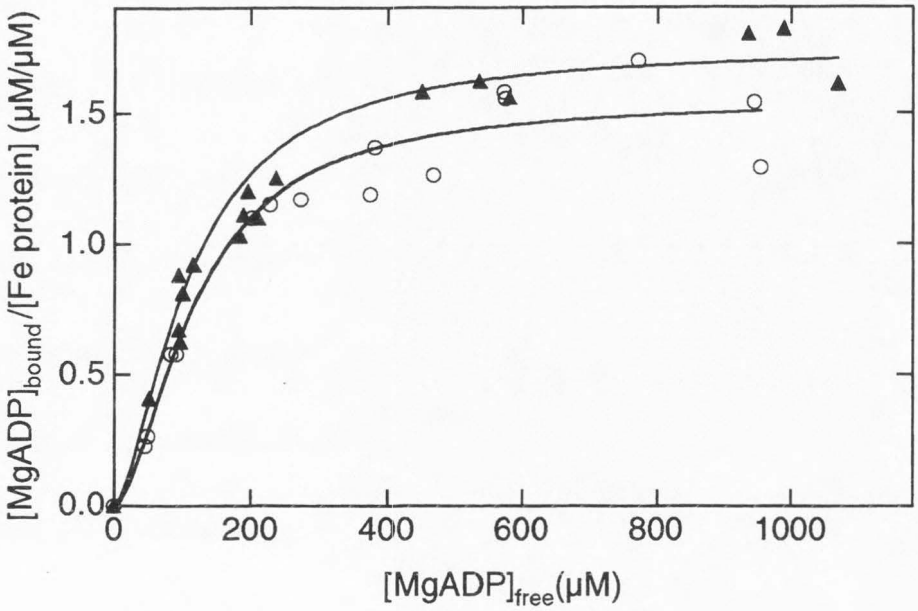

FIGURE 3-2: MgADP binding to the wild-type and K10R Fe proteins. Equilibrium column binding of MgADP to the wild-type and K10R Fe proteins was described in Experimental Procedures. The ratio of the concentration of MgADP bound to the Fe protein concentration was plotted against the free MgADP concentration. The data were fit to the Hill equation, $F_b=M_b[S]^n/(K_d+[S]^n)$, where F_b is the fraction of MgADP bound per Fe protein, M_b is the maximum number of MgADP bound per Fe protein, [S] is the free MgADP concentration, n is the cooperativity factor, and K_d is the apparent dissociation constant. Apparent K_d of 123μM with n=1.7 and M_b=1.7 was determined for MgADP binding to the wild-type Fe protein (▲). For MgADP binding to the K10R Fe protein (●), apparent K_d of 119 μM with n=1.7 and M_b=1.6 was calculated.

In order to compare the affinity of the K10R Fe protein for MgATP with that of the wild-type Fe protein, chelation experiments were performed and the results are shown in Figure 3-3. Upon addition of MgATP, Fe^{2+} can be chelated from the [4Fe-4S] cluster of the Fe protein by chelators such as BPS (26). The absorbance change at 534 nm due to the formation of the Fe^{2+}-BPS complex was recorded over time. The initial rate of absorbance change per second was calculated. The initial rate of chelation versus BPS concentration was plotted and the data were fit into the Hill equation. It was found that the K10R Fe protein has a dissociation rate constant (K_d) and Hill cooprativity number very similar to the wild-type Fe protein. Based on the results of the equilibrium column binding and the chelation experiments, the K10R Fe protein appears to bind MgADP or MgATP with similar affinity as the wild-type Fe protein. This implies that Lys 10 of the Fe protein probably is not involved in the nucleotide binding, which is considered to be the first step in nitrogenase catalysis.

Conformational Changes of the K10R and Wild-Type Fe Proteins upon Nucleotide Binding. Upon nucleotide (MgATP or MgADP) binding to the wild-type Fe protein, conformational changes induced in the Fe protein can be reflected as changes in the properties of the [4Fe-4S] cluster of the Fe protein. These changes include a shift in the midpoint potential of the $[4Fe\text{-}4S]^{2+/+}$ by over -100 mV and changes in the EPR and circular dichroism (CD) spectra of the Fe protein (7, 27-29). It has been shown that the

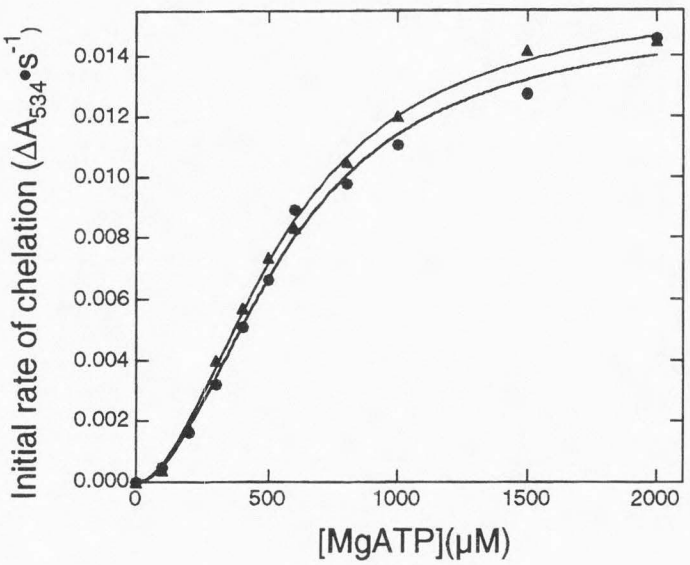

FIGURE 3-3: The initial rates of MgATP-dependent chelation of Fe^{2+} from [4Fe-4S] cluster of the Fe proteins by bathophenanthrolinedisulfonate (BPS) at different MgATP concentrations. The chelation experiment was performed as described in Experimental Procedures. At specific MgATP concentration, the absorbance of Fe^{2+} -BPS at 534 nm versus time was first recorded and the initial rate of absorbance change per second was calculated. The initial rate of chelation at different MgATP concentrations was plotted against the MgATP concentration. The data were fit to the Hill equation, $V=V_{max}[S]^n /(K_d+[S]^n)$, where V is the initial rate of chelation, V_{max} is the maximum rate of chelation , [S] is the MgATP concentration, n is the cooperativity factor, and K_d is the apparent dissociation constant. For the wild-type Fe protein (▲), apparent K_d of 550μM with n=1.9 and V_{max}=0.0159 were determined. For the K10R Fe protein (●), apparent K_d of 580 μM with n=1.9 and V_{max}=0.0152 was calculated.

Fe protein alone and the Fe protein in the presence of MgATP or MgADP give distinct CD spectra (27). Figure 3-4 shows the CD spectra of the wild-type and K10R Fe proteins in the absence and presence of nucleotides. The CD spectra of the K10R Fe protein have very similar features to those of the wild-type Fe protein. The midpoint potential and the EPR spectra of the wild-type and K10R Fe proteins both with and without nucleotides bound are determined in order to compare the nucleotide-induced conformational changes of the K10R Fe protein with those of the wild-type Fe protein (data not shown). The results support the idea that the K10R Fe protein undergoes similar conformational changes as the wild-type Fe protein upon nucleotide binding.

Primary Electron Transfer from the Fe Protein to the MoFe Protein. Given that interactions of nucleotides with the K10R Fe protein in the absence of the MoFe protein were similar to those of the wild-type Fe protein but the K10R Fe protein was inactive in substrate reduction, interactions of the K10R Fe protein with the MoFe protein were investigated. After the Fe protein binds to the MoFe protein, an electron is transferred from the Fe protein to the MoFe protein, an event that is coupled to MgATP hydrolysis in the Fe protein (3, 4). The absorbance increase at 430 nm due to the oxidation of the [4Fe-4S] cluster of the Fe protein from 1+ to 2+ state during electron transfer was monitored by stopped-flow spectroscopy. Figure 3-5 shows the increase in the absorbance upon time, which was fit to the single exponential equation. The apparent

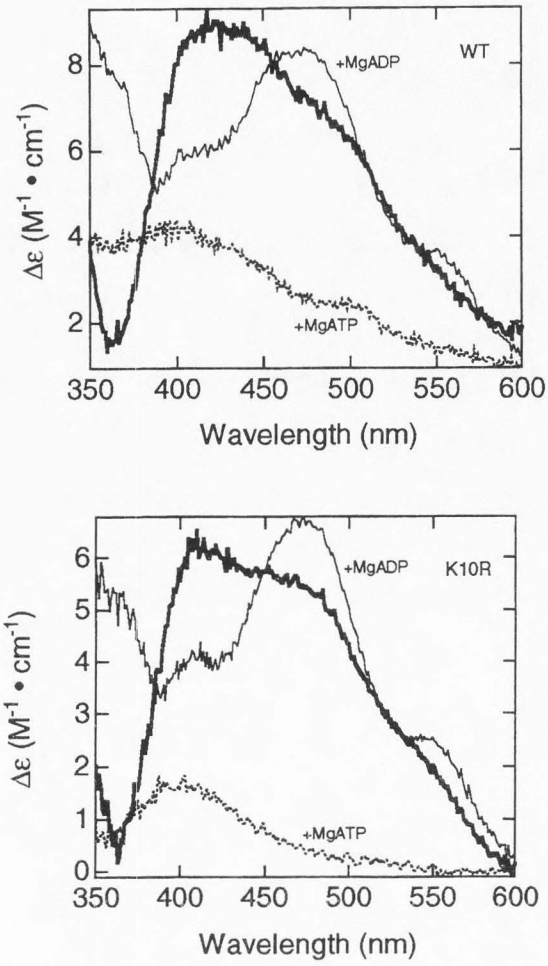

FIGURE 3-4: Circular dichroism spectra of wild-type and K10R Fe proteins with or without nucleotides bound. The visible circular dichroism (CD) spectra of oxidized Fe proteins were recorded as described under Experimental Procedures. All spectra were baseline subtracted. For the CD spectra of Fe protein in the presence of nucleotides, either MgATP or MgADP was added to a final concentration of 1 mM. The top panel and bottom panel show the CD spectra of wild-type or K10R Fe protein in the absence and presence of nucleotides, respectively.

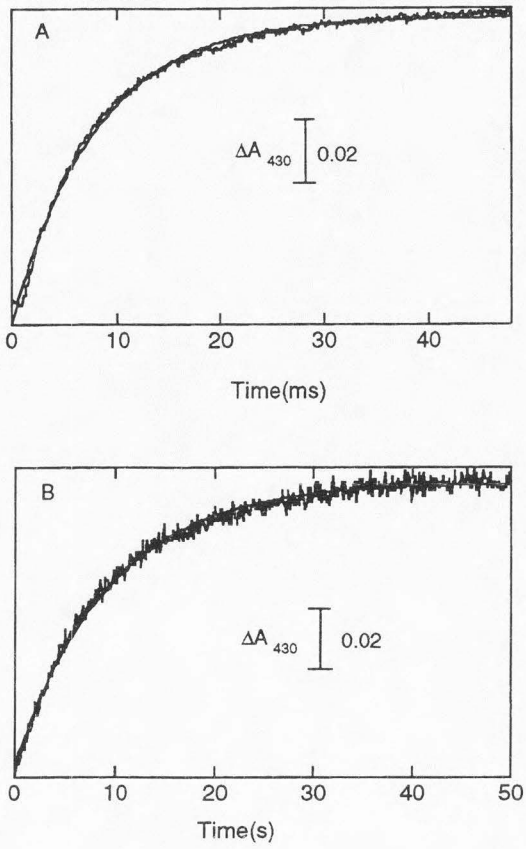

FIGURE 3-5: Pre-steady-state electron transfer from the wild-type or K10R Fe protein to the wild-type MoFe protein. The absorbance at 430 nm versus time was monitored by stoppped-flow spectrophotometry as described in Experimental Procedures. Panel A: After mixing a solution containing 80 μM wild-type Fe protein, 10 mM dithionite and 5 mM MgATP with a solution containing 20 μM wild-type MoFe protein and 10 mM dithionite, the absorbance change due to the oxidation of the [4Fe-4S] cluster of the Fe protein by the MoFe protein was monitored. The data were fit to a single exponential equation and the rate constant 124 s^{-1} was determined. Panel B: The procedures are the same as in the panel A except the 80 μM K10R Fe protein was used instead of the 80 μM wild-type Fe protein. The rate constant of 0.109 s^{-1} for the K10R Fe protein was determined after single exponential fits to the data.

first-order rate constant of 0.109 s^{-1} was determined for the K10R Fe protein, which is in contrast with the rate constant of 124 s^{-1} determined for the wild-type Fe protein under the same conditions.

Dissociation of the Wild-Type and K10R Fe Proteins from the Wild-Type MoFe Protein. Dissociation of the oxidized Fe protein from the MoFe protein is thought to be the rate-limiting step in nitrogenase catalysis (30). Stopped-flow spectrophotometry was used to determine the affinity of the K10R Fe protein with MgADP bound for the MoFe protein. The absorbance decrease at 430 nm due to the reduction of the oxidized K10R Fe protein by dithionite was monitored over time. The results are shown in Figure 3-6. Previous studies have shown that dithionite is not able to reduce the Fe protein that is bound to the MoFe protein (9). The rate constant determined in the experiment depends on the dissociation rate of the Fe protein from the MoFe protein and the reduction rate of the oxidized Fe protein by dithionite. The reduction rates of the oxidized K10R and wild-type Fe proteins by dithionite are very similar. Therefore, the rate constants determined in the experiment can be used to compare the dissociation rates of the K10R and wild-type Fe proteins from the MoFe protein. The dissociation rate constant of 5 s^{-1} was determined for the K10R Fe protein compared to 6 s^{-1} determined for the wild-type Fe protein.

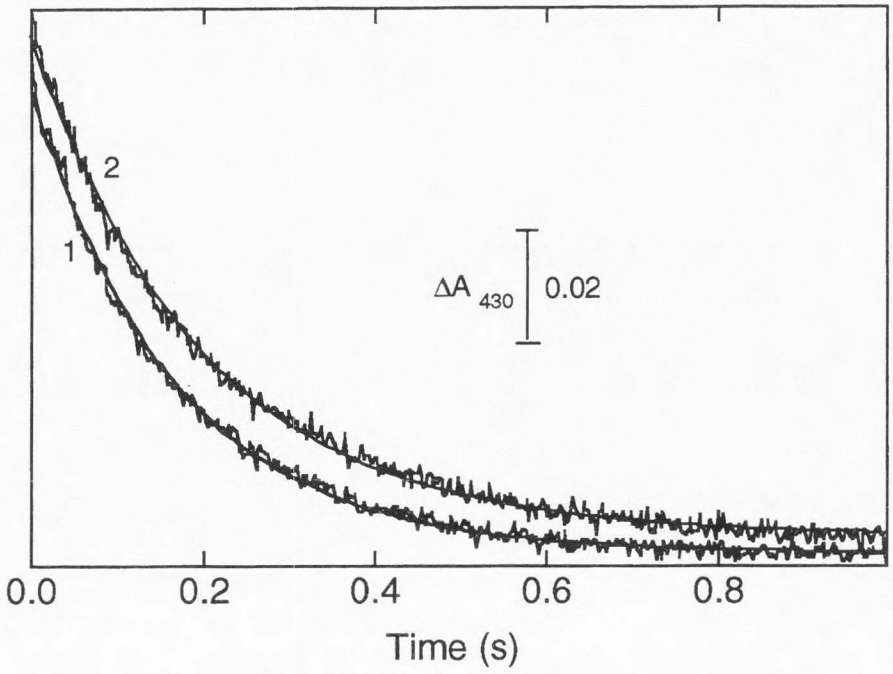

FIGURE 3-6: Dissociation of the wild-type or K10R Fe protein from the wild-type MoFe protein. The absorbance change at 430 nm was monitored by stoppped-flow spectrophotometry. After mixing a solution containing 40 μM IDS-oxidized wild-type Fe protein (trace1) or K10R Fe protein (trace 2), 40 μM dithionite-free reduced wild-type MoFe protein, 5 mM MgADP with a solution containing 200 μM wild-type Fe protein and 10 mM dithionite, the absorbance decrease at 430 nm over time was monitored due to the reduction of the [4Fe-4S] cluster of the Fe protein by dithionite upon dissociation from the MoFe protein in the presence of MgADP. After the data were fit to the single exponential equation, the rate constant of 6.01 s^{-1} for the wild-type Fe protein and the rate constant of 5.02 s^{-1} for the K10R Fe protein were calculated.

DISCUSSION

The mechanisms of nucleotide hydrolysis have been proposed for many nucleotide switch proteins such as G proteins, p21ras (31-33). During nucleotide hydrolysis, a specific amino acid residue will act as a general base to abstract a proton from the water. This activated water in the nucleotide binding site then attacks the γ-phosphate of nucleotide. Other amino acid residues near the binding site may serve to either stabilize the developed negative charge of the pentacovalent intermediate or facilitate the product formation. Significant reorientations of these catalytic residues have been observed in the formation of the transition state. For instance, in the crystal structure of $G_{i\alpha 1}$-GDP-AlF_4^- (31), it has been shown that a glutamine (Gln 204) can activate a water molecule in the nucleotide binding site for nucleophilic catalysis. A nearby arginine (Arg 178) is hydrogen bonded with two F atoms of the AlF_4^- and may function in stabilizing the negative charge of the transition state intermediate. The crystal structure of p21ras-GAP-GDP-AlF_4^- shows that Arg 778 of the GAP-334 (GTPase activating protein) is provided into the active site of the p21ras and helps stabilize the intermediate (33). A glutamine (Gln 61) of p21ras is proposed to act as a general base in the nucleotide binding site to abstract a proton from a water molecule, thus activating the water for catalysis. A similar mechanism of nucleotide hydrolysis has been proposed based on the studies of elongation factor-Tu (34).

The crystal structure of the ADP•AlF_4^- stabilized nitrogenase complex indicates that Lys 10 and Asp 129 of the Fe protein are involved in ATP hydrolysis in the nitrogenase complex (15). Lys 10 and Asp 129 both cross the subunit interface and interact with the nucleotide bound in the other subunit, while neither Lys 10 nor Asp 129 crosses the subunit interface in the crystal structure of the Fe protein alone. This suggests reorientations of these two residues upon transition state formation. The Asp 129 is in contact with the AlF_4^- probably through a bound water molecule (15) and has been proposed to function as a general base to activate the water molecule (16). Earlier studies have shown that changing Asp 129 to Glu resulted in an Fe protein that is not able to hydrolyze MgATP in the presence of the MoFe protein. The ε-NH_2 group of the Lys 10 is close to the F atoms of the AlF_4^- and a terminal oxygen of the β- phosphate. Lys 10 could either stabilize the pentacoordinate intermediate or the leaving group based on the crystal structure of the nitrogenase complex.

This study investigates the role of Lys 10 in the Fe protein using site-directed mutagenesis to change the codon for Lys 10 to the codon for Arg in the A. *vinelandii* Fe protein gene, *nif*H. Changing Lys 10 to Arg does not affect the affinity of the Fe protein for nucleotides. Characterization of the K10R Fe protein showed that the reactivity of the [4Fe-4S] cluster with the chelator upon MgATP binding to the K10R Fe protein, EPR, and CD spectra of the K10R Fe protein in the presence of nucleotides

are all similar to those of the wild-type Fe protein under the same conditions. It appears that Lys 10 of the Fe protein does not play a role in nucleotide-induced conformational changes prior to Fe protein-MoFe protein complex formation. Upon nitrogenase complex formation, the rate constant of electron transfer from the K10R Fe protein to the MoFe protein was 0.11 s^{-1} compared to 124 s^{-1} observed for the wild-type nitrogenase. In addition, the reductant-dependent MgATP hydrolysis rate of the K10R Fe protein was only 3% that of the wild-type Fe protein when the Fe protein was combined with the MoFe protein. The dramatic decrease in the ATP hydrolysis rate of the K10R Fe protein indicates that Lys 10 plays a role in MgATP hydrolysis in the nitrogenase complex. The results from this study indicate that Lys 10 interacts with nucleotides only after nitrogenase complex formation and imply that the Fe protein conformation with nucleotides bound is different from that within the nitrogenase complex. Substitution of Lys 10 with Arg in the Fe protein may not allow the altered Fe protein to efficiently stabilize the intermediate state of nucleotide hydrolysis. The oxidized K10R Fe protein was found to dissociate from the MoFe protein at a rate similar to that of the wild-type Fe protein, which supports that upon changing Lys 10 to Arg, the affinity of the oxidized Fe protein for the MoFe protein is not changed.

Because the Fe protein cannot hydrolyze ATP without the MoFe protein, it is reasonable that upon the Fe protein binding to the MoFe protein, the Fe protein will

undergo further conformational changes, which may cause Lys 10 and Asp 129 in one subunit to move across the subunit interface and serve as catalytic residues for MgATP hydrolysis in the other subunit. The switch I region in the Fe protein includes residues 39 to 67 and may play a role in MgATP hydrolysis that is initiated by the nitrogenase complex formation. Asp 39 is located close to the nucleotide binding site of the Fe protein, and the loop formed by residues 59 to 67 is thought to be important for the binding of the Fe protein to the MoFe protein. This switch I region has been suggested to function in communicating the Fe protein-MoFe protein docking surface and the nucleotide binding site of the Fe protein (3). Upon the Fe protein binding to the MoFe protein, the conformational changes induced in the Fe protein through switch I may propagate to result in a change in the positions of Lys 10 and Asp 129, thus stimulating ATP hydrolysis.

The Fe protein adopts a more compact conformation during the transition state of MgATP hydrolysis than that of the Fe protein in the absence of the MoFe protein (15). This compact conformation induced upon complex formation provides the structural basis for MgATP hydrolysis. As MgATP hydrolysis is coupled to electron transfer within the nitrogenase complex, it has been suggested that stabilization of this compact conformation during the transition state is also a prerequisite for electron tranfer to occur (15).

In summary, characterization of the K10R Fe protein revealed that changing Lys 10 to Arg in the Fe protein does not affect the nucleotide binding affinity of the Fe protein or the nucleotide-induced conformational changes in the Fe protein before the nitrogenase complex formation, but does dramatically decrease the MgATP hydrolysis rate of the nitrogenase. The results presented in this work support a role of Lys 10 of the Fe protein in MgATP hydrolysis after the Fe protein-MoFe protein complex formation.

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CHAPTER 4

SUMMARY

Some major questions regarding nitrogenase mechanism are: 1. How is ATP hydrolysis coupled to intramolecular electron transfer within the nitrogenase complex? 2. How is the electron delivered from the Fe protein to the P cluster of the MoFe protein and then to the FeMo cofactor? 3. Where do substrates, such as N_2, bind at the FeMo cofactor of the MoFe protein and how are those substrates reduced?

FE PROTEIN USES NUCLEOTIDE AS A SIGNAL TRANSDUCTION SWITCH

The research described in this thesis is focused mainly on the Fe protein of the nitrogenase. Nucleotide binding and hydrolysis in the Fe protein induce a series of conformational changes in the Fe protein, which initiates the events of nitrogenase catalysis. Other proteins that have nucleotide-dependent signal transduction mechanisms include G proteins, elongation factor-Tu, RecA, p21 Ras, myosin, etc. (1). Understanding the roles of nucleotides in nitrogenase catalysis will also contribute information as to the mechanism of other nucleotide binding proteins.

The crystal structure of the AlF_4^-•ADP stabilized nitrogenase complex provided information on the Fe protein structure in the nitrogenase complex and interactions

between the Fe protein and the MoFe protein (2). So far there is no crystal structure of the wild-type Fe protein with MgATP or MgADP bound. As MgATP tends to hydrolyze in the solution, efforts have been given to grow the Fe protein crystals in the presence of non-hydrolyzable ATP analogs, such as AppNHp and $AppCH_2p$, with the bridging oxygen of the β and γ phosphates of ATP substituted with the NH and CH_2 group, respectively. If we can get and compare the crystal structures of wild-type Fe protein without nucleotides, wild-type Fe protein with MgATP or MgATP analog bound, wild-type Fe protein with MgADP bound, and wild-type Fe protein in the nitrogenase complex, we can gain some insights into why nucleotide-induced conformational changes in the Fe protein are critical to substrate reduction on structural basis.

ROLES OF TWO ATP BINDING SITES OF THE FE PROTIEN IN NITROGENASE CATALYSIS

ATP plays a central role in nitrogenase catalysis. The Fe protein has two identical subunits with each subunit containing one ATP binding site. In order to assess the contributions of individual ATP binding sites in the nitrogenase catalysis, a heterodimeric Fe protein was constructed that has Asp 39 replaced by Asn in the ATP binding domain in one subunit, while the other subunit retains Asp 39 as in a wild-type

Fe protein subunit. The properties of the [4Fe-4S] cluster of the heterodimeric Fe protein upon nucleotide binding were between those of wild-type homodimeric Fe protein [Asp^{39}/Asp^{39}] and altered homodimeric Fe protein [Asn^{39}/Asn^{39}]. These results indicate that changes in the properties of the [4Fe-4S] cluster of the Fe protein affected by nucleotide binding are the result of additive conformational changes contributed by each subunit. If the crystal structure of the heterodimeric [Asp^{39}/Asn^{39}] Fe protein in the presence of MgATP or MgADP is available and compared with those of the wild-type Fe protein and homodimeric [Asn^{39}/Asn^{39}] Fe protein with the same kind of nucleotide bound, more information will be provided as to how the protein conformational changes affect the properties of the [4Fe-4S] cluster of the Fe protein.

The MgATP-dependent electron transfer rate of the heterodimeric [Asp^{39}/Asn^{39}] Fe protein was 2.2 s^{-1}, which is between the rates observed for the wild-type, approximately 100 s^{-1}, and [Asn^{39}/Asn^{39}] homodimeric Fe proteins, 0.02 s^{-1}. The increasing trend in the electron transfer rates for the homodimeric and heterodimeric Fe proteins supports the idea that each subunit of the Fe protein contributes to the rate acceleration of primary electron transfer.

The heterodimeric [Asp^{39}/Asn^{39}] Fe protein, like the altered homodimeric Fe protein [Asn^{39}/Asn^{39}], forms a tight complex with the MoFe protein after electron transfer while the wild-type Fe protein does not. Alteration of one subunit of the Fe

protein is sufficient for the formation of a tight complex. It is possible that the Fe protein interacts with the pseudosymmetric MoFe protein in a certain orientation. The tight complex formed by this heterodimeric Fe protein [Asp^{39}/Asn^{39}] and the MoFe protein may account for the inability of this nitrogenase to reduce substrates, which implied that two subunits of the Fe protein are required to function properly for the substrate reduction.

MGATP HYDROLYSIS INITIATED BY FE PROTEIN-MOFE PROTEIN COMPLEX FORMATION

Another area described in this thesis is the mechanism of nucleotide hydrolysis in nitrogenase or how the protein complex formation stimulates nucleotide hydrolysis. The crystal structure of the AlF_4^-•ADP-stabilized nitrogenase complex shows that Lys 10 and Asp 129 of the Fe protein both cross the subunit interface and interact with the bound nucleotide in the other subunit (2) while neither Lys 10 nor Asp 129 crosses the subunit interface in the crystal structure of the Fe protein alone (3). As the Fe protein is not able to hydrolyze ATP without the MoFe protein, it seems reasonable to speculate that binding of the Fe protein to the MoFe protein induces further conformational changes in the Fe protein, which will cause the reorientations of catalytic residues, like Lys 10 and Asp 129, in the Fe protein for MgATP hydrolysis.

The crystal structure of the transition-state nitrogenase complex shows that Asp 129 interacts with AlF_4^- probably through a bound water molecule and this residue could function as a general base to activate a water molecule for MgATP hydrolysis. The role of Asp 129 was supported by earlier work showing that changing Asp 129 to Glu resulted in an Fe protein that cannot hydrolyze MgATP when combined with the MoFe protein (4). Another potentially catalytic residue, Lys 10, is close to the AlF_4^- and β-phosphate, and the position of Lys 10 allows it to stabilize the negative charge of the pentacoordinate phosphate intermediate or leaving group. The Fe protein with Lys 10 substituted with Arg was constructed to further investigate the role of Lys 10 in the Fe protein. Characterization of the K10R Fe protein shows that Lys 10 is not involved in the ATP or ADP binding to the Fe protein. In addition, Lys 10 of the Fe protein does not play a role in the nucleotide-induced conformational change prior to Fe protein-MoFe protein complex formation. After nitrogenase complex formation, the reductant-dependent MgATP hydrolysis rate of the K10R Fe protein is only 3% of that of the wild-type Fe protein. The dramatic decrease in the ATP hydrolysis rate of the K10R Fe protein indicated a role of Lys 10 in MgATP hydrolysis within the nitrogenase complex.

HOW THE NITROGENASE UTILIZES THE ENERGY OF NUCLEOTIDE BINDING AND HYDROLYSIS TO REDUCE SUBSTRATES

Although the reduction of the N_2 to ammonia is thermodynamically favorable, the formation of the intermediate states during substrate reduction could be unfavorable. The energy associated with MgATP binding and hydrolysis may be used to stabilize the specific protein conformations and control electron transfer required for substrate reduction catalyzed by nitrogenase (5). The construction of heterodimeric Fe proteins will allow further studies to address some of these areas regarding nitrogenase mechanism.

A potentially interesting heterodimeric Fe protein would be [Lys^{15}/Arg^{15}] Fe protein. The homodimeric-altered [Arg^{15}/Arg^{15}] Fe protein, or K15R Fe protein, is known to be unable to bind nucleotides (6). With this heterodimeric [Lys^{15}/Arg^{15}] Fe protein, one could ask a question: how does one subunit with the ability to bind the nucleotide affect the other subunit which is unable to bind the nucleotide? Characterization of this protein will provide information on the cooperativity of nucleotide binding to the Fe protein. As the energy provided by two MgATP binding to the Fe protein is responsible for the lowering of the midpoint potential of the Fe protein by over 100 mV (4), if this heterodimeric Fe protein only binds one nucleotide, it will

be interesting to see if it can still undergo nucleotide-induced conformational change and ATP hydrolysis-coupled electron transfer.

MgATP hydrolysis is coupled to the electron transfer event in the nitrogenase complex. The ATP/e^- ratio has been reported to vary from 1 to 2.5, depending on the experimental conditions used (7). It is not clear whether the two bound MgATP in the Fe protein are hydrolyzed at the same time or sequentially upon the nitrogenase complex formation. Is the energy associated with two MgATP hydrolysis during one catalytic cycle required for nitrogenase catalysis? One way to address this question is to construct a heterodimeric Fe protein that can only hydrolyze one bound MgATP during one catalytic nitrogenase cycle. An altered homodimeric Fe protein with Lys 10 substituted with Arg was found to hydrolyze MgATP at a rate 3% that of the wild-type Fe protein. It is possible that changing Lys 10 to neutral or negatively charged residue will result in a homodimeric-altered Fe protein that has no activity to hydrolyze MgATP. Then a heterodimeric Fe protein could be constructed, with one subunit that is the same as this altered homodimeric Fe protein subunit, and the other subunit that remains unchanged as the wild-type Fe protein subunit. Given that Lys 10 of the Fe protein is not involved in the nucleotide-induced conformational changes, this heterodimeric Fe protein should associate with the MoFe protein and may only

hydrolyze one MgATP. The electron transfer event and the substrate reduction activity of this heterodimeric Fe protein can be investigated.

ROLE OF THE "CLOSED" CONFORMATION OF THE FE PROTEIN IN ELECTRON TRANSFER AND MGATP HYDROLYSIS

How nucleotide hydrolysis is coupled to electron transfer is not well understood. Comparison of the crystal structures of the wild-type Fe protein alone and the wild-type Fe protein in the nitrogenase complex shows that upon complex formation, the Fe protein subunits rotate about 13° toward the subunit interface and this rotation causes the Fe protein to be in a more compact or "closed" conformation that can be reflected as a decrease of the gyrational radius of the Fe protein (2). It is possible that ATP helps stabilize this closed conformation of the Fe protein, thus facilitating electron transfer from the Fe protein to the MoFe protein. In order to test whether or not this closed conformation of the Fe protein is required for electron transfer, an altered Fe protein, which is unable to form the closed interface between the Fe protein subunits, needs to be constructed by means of site-directed mutagenesis to change specific residues that are involved in the intersubunit interactions after the Fe protein-MoFe protein complex formation. The ability of this altered Fe protein to transfer electrons then can be investigated.

Upon association of the Fe protein with the MoFe protein, conformational changes induced in the Fe protein are possibly mediated by switch I in the Fe protein. Switch I in the Fe protein extends from residues 39 to 67, where Asp 39 is located close to the nucleotide binding site of the Fe protein and the loop formed by residues 59 to 67 is thought to be involved in the binding of the Fe protein to the MoFe protein. The closed conformation of the Fe protein induced upon nitrogenase complex formation could be a prerequisite for ATP hydrolysis and electron transfer to occur. If an altered Fe protein is constructed that can somehow achieve this compact conformation by deletion of some residues in switch I, will this protein still bind nucleotides and exchange nucleotides? If this Fe protein does, can it hydrolyze MgATP in the absence of the MoFe protein? The roles of switch I in the Fe protein need to be further investigated.

ROLE OF THE ADENINE PART OF ATP IN NITROGENASE CATALYSIS

For the wild-type nitrogenase, hydrolysis of other nucleotides except ATP is not coupled to electron transfer from the Fe protein to the MoFe protein. It was found that the interactions of the adenine portion of ATP with the protein residues are important for nucleotide-induced conformational changes (8). The interactions of the Fe protein with the adenine part of ATP could influence the properties of the [4Fe-4S] cluster by

directly inducing protein conformational changes or affecting the positions of the ribose or the phosphate moieties of ATP. The crystal structure of the AlF_4^-•ADP-stabilized nitrogenase complex shows that the main chain amide of Asp 214 forms a hydrogen bond with the N-1 of the adenine, while the exocyclic amino group of the adenine interacts with the main chain oxygen of Pro 212 and the side chain of Asn 185 (2). These interactions may determine the specificity of the Fe protein for ATP. Asp 214, Asn 185, and Pro 212 can be targets of site-directed mutagenesis in order to gain some insights into why ATP is specifically required for reduction of substrates catalyzed by nitrogenase.

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APPENDIX

Utah State
UNIVERSITY

June 9, 2000

Dr. Noelle Cockett
Interim Dean
School of Graduate Studies
Utah State University
Logan, UT

Dear Noelle,

This letter is a follow up to our phone conversation this morning regarding the thesis of Ms. Wei Wu, a candidate for the MS degree in Biochemistry. In Ms. Wu's thesis, chapter 2 presents only work done by her. This is only part of the whole body of work contained in the publication in *Biochemistry* authored by Chan, Wu, Dean, and myself. The contributions of Ms. Chan will be presented as a chapter in her dissertation as part of her requirements for the Ph.D. in Biochemistry.

I hope that this clarifies your questions. Please let me know if I can be of further help.

Lance Seefeldt
Associate Professor

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